Genetic Determinants of Texture in Strawberry Fruits

By Aram Buchanan, BSc MSc

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ABSTRACT

Fruit texture is a quantitative trait believed to be determined by a number of genetic loci. In order to identify genes in strawberries that are specifically associated with texture a cross was made between a cultivar with firm fruits and a cultivar with soft fruits. The resultant population of 269F_1 plants produced ripe fruits whose firmness values differed over a five-fold range. Pools of twenty plants having either the softest or firmest fruits were analysed by two methods. The first, suppressive subtractive hybridisation initially indicated that many genes were differentially expressed in fruits from both the firm and soft pools. Differential hybridisation of 80 subtracted cDNA clones from each pool with the cDNA subtracted from each pool showed that many of the cDNAs were common to firm and soft fruit. When the subtracted cDNAs were hybridised with unsubtracted cDNA several clones from each pool appeared to be enhanced. However, northern analysis was unable to confirm these data. The second method used cDNA-AFLP to detect differentially expressed genes in the pools of firm and soft fruit. Using one set of amplification primers, bands corresponding to 27 cDNA fragments specific to either firm or soft fruit pools were resolved on a polyacrylamide gel. These fragments were cloned and sequenced but they had no obvious homology with genes associated with cell wall metabolism. Northern anlaysis indicated that the cloned cDNA-AFLP fragments represented polymorphisms unrelated to texture. One cDNA clone, AFLP-S13, appeared to have higher levels of the corresponding transcript in fruits from the soft parent and soft pool. Although this clone was upregulated during ripening its expression was

neither fruit specific nor correlated with texture. The expression of genes associated with cell wall metabolism including *cel*1, *cel*2 and expansin was also investigated in the segregating population.

LIST OF ABREVIATIONS

2-BE	-	2-butoxyethanol
2D-PAGE	-	Two-dimensional polyacrylamide gel electrophoresis
ACC	-	1-aminocyclopropane-1-carboxylic acid
ACP	-	Acyl carrier protein
BAC	-	Bacterial Artificial Chromosome
CAD	-	Cinnamyl Alcohol Dehydrogenase
cDNA	-	complementary Deoxyribonucleic acid
DEPC	-	Diethyl pyrocarbonate
CHAPS	-	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CHS	-	Chalcone synthase
DD	-	Differential Display
DD-RT-PCR	-	Differential Display Reverse Transcriptase PCR
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DS	-	Differential screening
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
EGase	-	Endoglucanase
ER	-	Endoplasmic Reticulum
EST	-	Expressed sequence tag
F3H	-	Flavanone 3-hydroxylase
G3PDH	-	Glyceraldehyde-3-phosphate dehydrogenase
gf	-	Greenflesh
icx	-	Irregular xylem
IDH	-	Isocitrate Dehydrogenase
IPG	-	"Immobiline® DryStrip"
IPTG	-	Isopropyl β-D-thiogalactopyranoside

JA	-	Jasmonic Acid
MeJA	-	methyl ester Jasmonic Acid
MOPS	-	3-(N-Morpholino)propanesulfonic acid
mRNA	-	messenger Ribonucleic acid
nr	-	Never-ripe
nor	-	Non-ripening
PAL	-	Phenylalanine Ammonia Lyase
PCR	-	Polymerase Chain Reaction
PG	-	Polygalacturonase
PME	-	Pectinmethylesterase
QTL	-	Quantative Trait Locus
rin	-	Ripening inhibitor
RNA	-	Ribonucleic acid
RNAse	-	Ribonuclease
RT-PCR	-	Reverse Transcriptase PCR
SDS	-	Sodium Dodecyl Sulfate
SH	-	Subtractive Hybridisation
SSH	-	Suppressive Subtractive Hybridisation
TCA	-	Trichloro Acetic Acid
TDF	-	Transcript Derived Fragment
TYLCV	-	Tomato Yellow Leaf Curl Virus
XET	-	Xyloglucan Endotransglycosylase
X-gal	-	Bromo-4-chloro-3-indolyl β-D-galactoside
YAC	-	Yeast Artificial Chromosome

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Chapter 1: INTRODUCTION

1.1 STRAWBERRY FRUIT

The strawberry (*Fragaria x ananassa*) is an economically important fruit crop with a world production of 3 million metric tones in 1999 (FAO, 1999). The strawberry is classified as a soft fruit and as such is very easily damaged during harvesting, transportation and storage. Improving the post-harvest life of strawberries has received much attention. Larsen and Watkins (1995) for example showed that storing the fruit at 0°C and in an atmosphere of 20% (w/v) CO_2 improved its shelf life and minimised the development of off-flavours. Delays in cooling fruit after harvest had a negative effect on appearance, nutritional quality and marketability (Nunes, 1995). Another postharvest treatment is to heat treat the harvested fruit which inhibits fungal development and slows the ripening rate (Civello *et al.*, 1997). Foliar application of $CaCl_2$ does prolong shelf life of strawberries, but the magnitude of the effect is dependent on the cultivar type and the calcium content of the fruit at the time of treatment (Chéour *et al.*, 1991). Environment factors can be manipulated to modulate ripening but their effectivness is ultimately limited by genotype. Further improvements, however will require a greater understanding of the underlying biochemistry of ripening and the genetic factors that regulate it.

Strawberry fruit grow rapidly after fertilisation and reach a maximum size after about 30 days depending on environmental conditions and cultivar type, factors that also affect softening (Kader, 1991). The ripening of strawberry fruit is characterised by an increase

in fresh weight and anthocyanin content and a decrease in fruit firmness (Abeles and Takeda, 1990). A study of cell wall metabolism in strawberry was made by Knee *et al.* (1977). Cell division in the developing fruit ceased after 7 days and fruit growth thereafter was mainly due to cell expansion. During ripening, the cell walls swell due to hydration and begin to separate as the polysaccharides in the middle lamella and cell wall matrix become more soluble (Knee *et al.*, 1977). The role of enzymes in cell wall degradation during strawberry fruit ripening is not fully characterised. Polygalacturonase (PG), an enzyme that has received much attention in the softening of tomato fruit (Slater *et al.*, 1985), has very little activity in fruit of strawberry (Huber, 1984, Abeles and Takeda, 1990). Pectinmethylesterase (PME) has been detected in strawberry and it has been reported that the activity of this enzyme rises to a maximum from unripe green fruit to the ripe stage but decreases in overripe fruit. (Barnes and Pratchett, 1976). These authors detected cellulase in ripe strawberry fruit and Abeles and Takeda (1990) reported an increase of cellulase activity during ripening. Although there are temporal correlation's between fruit ripening and increases in cellulase activity, it still has to be shown that there is a breakdown of native cellulose by cellulase. The term endoglucanase (EGase) is now used to distinguish plant cellulases from bacterial cellulases. Other cell wall proteins including expansins and pectate lyase may also have roles in cell wall disassembly (discussed in greater detail below).

1.2 FRUIT RIPENING

1.21 Respiration

Fruit ripening is usually associated with the synthesis of new proteins, mRNA, colour and flavour compounds. Differences in respiratory activity have enabled fruits to be separated into two categories classified either as climacteric or non-climacteric. Climacteric fruits, such as tomato, apple, banana and avocado, have a characteristic peak in respiratory activity during ripening. The respiration peak varies in magnitude and timing between fruits and the respiration rate correlates with the speed of ripening. Banana and avocado ripen rapidly and have high respiratory rates (Tucker, 1993). Non-climacteric fruits, such as strawberry, grape, cherry and lemon do not exhibit a respiration peak, but instead respiration declines during ripening. Again as with increased respiration in climacteric fruit, the decrease in respiration among the non-climacteric fruits is variable. The role of the climacteric response is unclear as non-climacteric fruit can ripen without an increase in respiration. It is thought that the peak in respiration in climacteric fruits is related to metabolic activity both of which correlate with ethylene production which also increases in climacteric fruit. Non-climacteric fruit, however, are unresponsive to ethylene.

1.2.2 Flavour and pigment changes

The flavour perception of fruit is determined by taste and smell. The taste of fruits is dependent upon the combination of sugars and organic acids and the overall flavour is complemented by volatiles and phenolics. The bulk of the sugar and organic acids in fruit is provided by the photosynthetic activity in the leaves. Some fruits such as banana and tomato assimilate most of their carbohydrates before ripening while other plants such as strawberry and grape accumulate sugars during ripening. Fruits that accumulate their sugars before ripening can be harvested early and ripened off the plant whereas strawberries and grapes will only ripen fully while attached to the plant.

In many fruits during ripening, starch is broken down to glucose, fructose or sucrose. In banana, α-amylase, which hydrolyses the α(1-4) linkages of amylose to produce a mixture of glucose and maltose, is more active than β-amylase and starch phosphorylase but all increase in activity during ripening. In unripe fruit of the of buttercup squash, α– and β-amylase activities are low while there is a rapid synthesis of starch, but during ripening the activities of these enzymes increase and this correlates with an increase in maltose content (Irving *et al.*, 1999). The sugars in strawberry fruit are mostly sucrose, glucose and fructose (Makinen and Söderling, 1980). Sucrose levels in strawberry fruit increase rapidly during fruit development and reach a maximum at the turning stage before decreasing in overripe fruit reference therein.

The mixture of volatiles that make up the aroma of fruits is very complex. In apple and orange fruit, around 230 and 330 different compounds, respectively make up the flavour profile (Tucker, 1993) and in strawberry about 200 compounds make up the aroma profile (Latrasse, 1991). Such a large number of compounds, however, may result from the action of a relatively small number of enzymes if they have overlapping substrate specificity (Manning, 1993).

During ripening, most fruit change colour although fruits such as apples and pears retain much of their green colour in the form of chlorophyll. Colour changes in fruit can occur by degradation of chlorophyll to reveal other pigments or by the formation of new compounds such as anthocyanins and carotenoids (Tucker, 1993). In tomato the unripe fruit is green and during ripening changes to red. The tomato ripening mutant, greenflesh (*gf*) has ripe fruits which are a muddy brown colour, due to impaired chlorophyll degradation. Carotenoid synthesis is normal in these fruits, indicating that the two pathways may not be directly related (Darby *et al.*, 1977).

The red colour of strawberry fruit is due to water-soluble anthocyanins. The anthocyanins are derived from the amino acid phenylalanine. Two important enzymes in anthocyanin biosynthesis are phenylalanine ammonia lyase (PAL) and chalcone synthase. A temporal relationship between PAL activity and anthocyanin accumulation was reported by Given *et al.* (1988b).

1.3 RIPENING MUTANTS

Ripening mutants have greatly helped in understanding the ripening process as the mutations affect several genes that regulate the ripening process. The most studied ripening mutants are those from tomato. These fall into two main groups, the first includes mutants which affect either chlorophyll metabolism, such as greenflesh (see above), or carotenoid biosynthesis such as yellowflesh and tangerine. The mutant cultivar of the cultivated strawberry *Fragaria ananassa*, "White Carter", lacks the red pigment anthocyanin when fully ripe. However, other ripening processess are unaffected such as texture and flavour (Manning, 1993). The second group have mutations, termed pleiotropic, that affect all aspects of fruit ripening. Ripening inhibitor (*rin*) and the non-ripening (*nor*) are severe mutants, which affect fruit colour, flavour, softening and ethylene production. Mature *rin* fruit are hard and are pale yellow in colour and are not affected by exogenous ethylene (Gray, 1994); *nor* fruit produce some lycopene during senescence and there is a slight increase in ethylene production (Brady, 1987). The ripening-impaired tomato mutant Never-ripe (*Nr*) is insensitive to the plant hormone ethylene. This phenotype is thought to occur due to a mutation in an ethylene receptor (Wilkinson *et al.*, 1995b). A new tomato mutant (*Cnr*) described by Thompson *et al.* (1999), has reduced ethylene production, an inhibition of softening, a yellow skin and a severe reduction in cell-to-cell adhesion. The phenotype of this mutant could not be reversed by exposure to exogenous ethylene. The gene has been mapped to chromosome 2.

The early molecular studies of fruit ripening used *in vitro* translation to identify mRNAs that were ripening related. The pTOM series of ripening related cDNA clones were isolated by differentially screening a ripe fruit cDNA library with cDNA from ripe fruit and and unripe fruit (Slater *et al.*, 1985). Several of the pTOM cDNAs were fruit specific and ethylene sensitive (Maunders *et al.*, 1987). Knapp *et al* (1989) reported that a number of pTOM cDNAs were present at much reduced levels during ripening of *rin* mutant fruit, although Southern blotting showed the presence of these genes. Picton *et al.* (1993) identified five novel ripening cDNAs, which showed reduced accumulation in *rin* mutant fruit. These cDNAs were isolated by screening a cDNA library, prepared from tomato fruit at the early stage of ripening, with cDNA from the *rin* mutant, prepared from fruit at a similar stage. The first ripening cDNA to be fully characterised was that of the cell wall degrading enzyme polygalacturonase. This was achieved by isolating the enzyme and comparing its amino acid sequence with the pTOM clones (Gray, 1994). Pectinmethylesterase was identified by screening the pTOM library with oligosaccharide probes derived from a tomato PME amino acid sequence. Antisense inhibition has been used to confirm the identity of pTOM13 as an ACC oxidase (Hamilton *et al.*, 1990) and pTOM5 as phytoene synthase (Bird *et al.*, 1991).

1.3 CHANGES TO FRUIT TEXTURE DURING RIPENING

1.4.1 Cell Wall Structure

The currently proposed model for plant cell walls envisages groups of cellulose polymers bonded to form semicrystalline microfibrils that are coated with hemicellulose and embedded in a matrix of pectin polysaccharides (Fig. 1.1) (Carpita and Gibeaut, 1993). The cellulose microfibrils consist of β(1-4)-linked glucose residues that form close lateral fibrils. Hemicelluloses have a carbohydrate backbone similar to cellulose resulting in strong hydrogen bonds between the two polymers (Reiter, 1998). Hemicelluloses comprise a variety of polymers including xyloglucans, glucomannans and galactomannans (Tucker, 1993). Xyloglucans are the main hemicellulose in dicot plants, and have a backbone of β(1-4)-linked glucosyl residues. The xylogucan backbone is modified by the addition of di- and trisaccharide side-chains, which can alter the physical properties of the polymers (Rose and Bennett, 1999). The pectin matrix is made up of a mixture of neutral pectins, arabinans, galactans or arabinangalactans and acidic pectins, rhamnogalacturonans and homogalacturonans. Rhamnogalacturonans are composed of β-(1-4)-linked glacturonic acid residues interspersed with rhamnose (Tucker, 1993). It is thought that galactose or arabinose side chains can attach to the rhamnose units. The molecules within the three dimensional network are held together by a mixture of covalent and non-covalent bonds (Fry, 1986). The degree of intermolecular bonding may determine the mechanical properties of the cell wall that affect fruit texture. The cellulosic regions of the cell wall are capable of

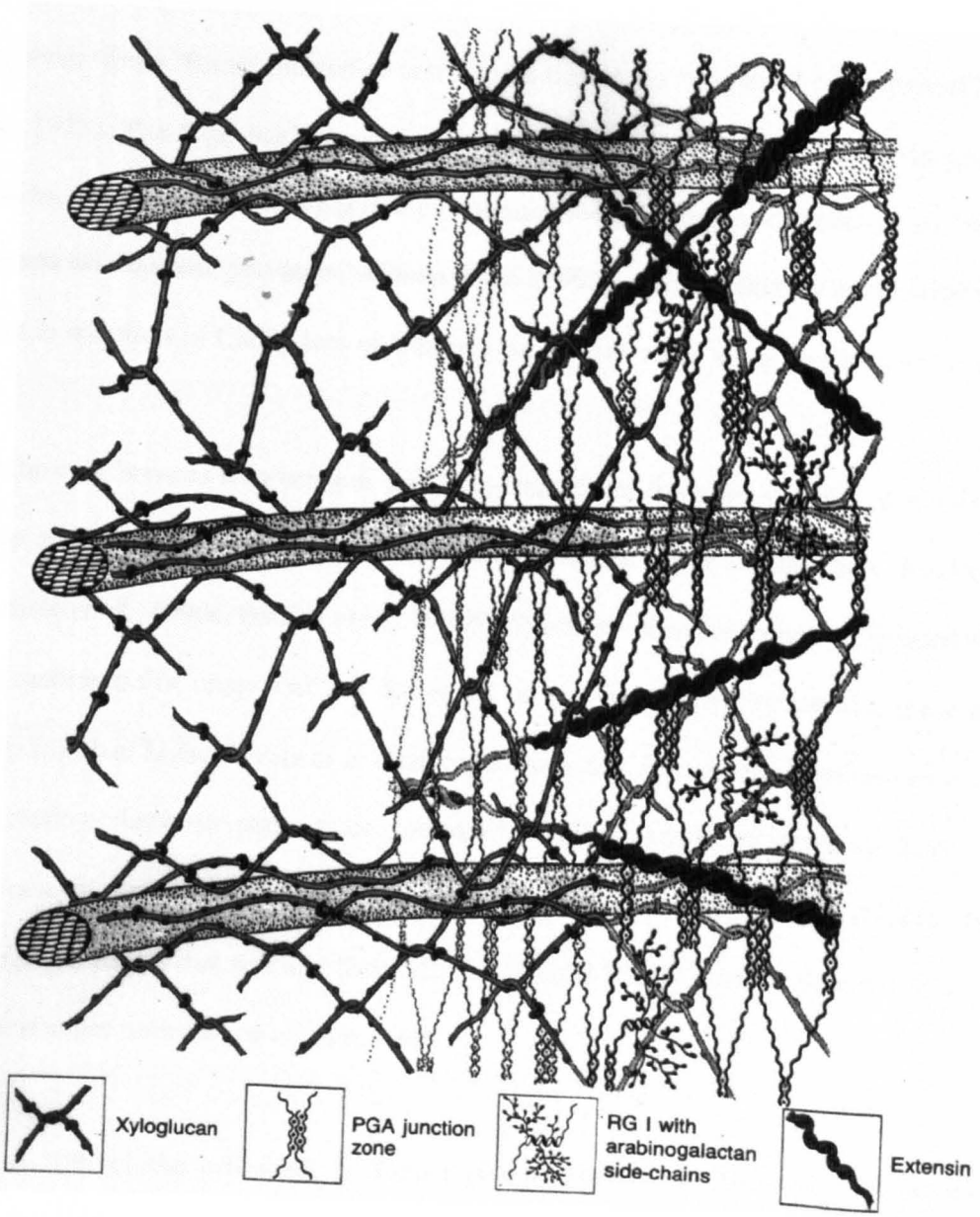

Figure 1.1 A model of the growing plant cell wall from Carpita and Gibeaut (1993)

inter-chain association via hydrogen bonding. Hayashi (1989) has postulated that as well as coating the cellulose microfibrils, hemicelluloses may also form bridges between adjacent fibrils. Pectin molecules can also be linked via "egg-box" complexes (Grant *et al.*, 1973). The "egg-box" structure is formed between de-esterfied polygalacturanosyl chains, which are linked by hydrated calcium ions binding co-operatively to carboxyl groups on adjacent polymers. Alonso *et al.* (1995) reported that by immersing cherry fruit in solutions of $CaCl_2$, loss of firmness can be prevented when the fruit is frozen.

Texture differences between soft and firm fruits from the same cultivar of cherry have been shown to be due to differences in cell wall turnover during their development (Batisse *et al.*, 1996a, Batisse *et al.*, 1996b). Synthesis of neutral sugars increased during the maturation of crisp fruit but decreased in soft fruit. At post-maturity, the walls of crisp fruit had higher levels of neutral sugars than soft fruit, so the possibilities of more interactions between pectins are increased. During strawberry ripening there is an increase in neutral sugars, such as rhamnose, arabinose and galactose (Huber, 1984). One possibility is that soft and firm cultivars of strawberries could have different rates of neutral sugar turnover, as in cherry fruit.

Up to 10% of the cell walls of higher plants comprise of proteins, with no known enzymatic function which are immobilised within the wall by covalent crosslinks (Reiter, 1998). The best known cell wall structural proteins are extensins, which are encoded by a multigene family that have cell specific expression patterns (Reiter, 1998). It is thought that extensins may be involved in strengthening the cell wall in response to

injury as studies have shown that extensin genes are induced in response to wounding and pathogen attack (Showalter, 1993, Jose and Puigdomenech, 1993).

The majority of research into cell wall structure has been carried out on primary cell walls. The 3-dimensional structure of fruit cells is not thought to differ markedly from that of cells of other tissues. Interestingly fruit cell walls seem to be relatively rich in pectin (Tucker, 1993). Just as ripening mutants and transgenic plants have helped in defining the general mechanism of fruit ripening, they have also helped in the molecular analysis of cell wall components. Some genes involved in the synthesis of cell wall glycans have been cloned and described including UDP-D-glucose 4-epimerase (Dormann and Benning, 1996), UDP-D-glucose dehydrogenase (Tenhaken and Thulke, 1996) and GDP-D-mannose 4,6-dehydrogenase (Bonin *et al.*, 1997). These genes are involved in nucleotide sugar interconversions at the early stage of cell wall synthesis and could determine how the cell wall is constructed (Reiter, 1998). Pear *et al.* (1996) reported the cloning of two cDNAs (*CelA*1 and *CelA*2) from cotton, which have homology to cellulose synthases present in bacteria such as *Agrobacterium tumefaciens*. *CelA*1 and *CelA*2 are highly expressed during the cellulose synthesis phase of cotton development. The CelA1 protein has three putative transmembrane domains indicating that it may be located in the plasma membrane where cellulose synthesis is thought to take place (Pear *et al.*, 1996).

Cellulose deficient mutants have been identified in chemically mutagenised *Arabidopsis*. The *icx* (irregular xylem) mutants display abnormal wall structures in cell types that

normally synthesise a thick secondary wall in infloresence stems (Turner and Somerville, 1997). Compared to wild type plants, the mutant plants have a five-fold lower cellulose content, whereas other cell wall polysaccharides are at comparable levels. Lignin content and distribution were similar to the wild-type plants, which suggest that the *icx* mutant allele codes for either a cellulose synthase or a regulatory gene, either of which may control cellulase synthesis.

Another study found eleven *Arabidopsis* mutants in a chemically mutagenised population, which had abnormal monosaccharide compositions (Reiter *et al.*, 1997). Plants that had the mutant *mur*1 locus were completely deficient in L-fucose in shoot derived material (Reiter *et al.*, 1993). The L-fucose was replaced by L-galactose (Zablackis *et al.*, 1996). The *mur*1 gene, encoding GDP-D-manose 4,6-dehydrogenase in *Arabidopsis* appears to have two isoforms, each encoded by separate genes (Reiter, 1998). Other *mur* mutants were not completely deficient in any monosaccharide, but either had significantly lower levels of a single monosaccharide such as rhamnose, fucose and arabinose or had more complex changes in monosaccharide composition (Reiter, 1998). The complexity of the plant cell wall indicates that many genes may encode enzymes or regulatory proteins involved in cell wall synthesis. The use of cell wall mutants and transgenic plants will help to decipher how the cell wall is synthesised and laid down.

1.4.1.2 Ferulate cross-linking in plant cell walls

Ferulic acid is esterified to the C5-hydroxyl of a-L-arabinose moieties of grass xylans (Mueller-Harvey *et al.*, 1986). There is strong evidence that ferulic acid containing cell-wall polysaccharides play an important role in maintaining the thermal stability of cell-wall adhesion and thus texture of chinese water chesnut (Parker and Waldron, 1995). Analysis of Chinese water chesnut revealed that 40% of feulic acid was in the form of six dimers that are capable of formingheat-stable crosslinks between polysaccharides (Parr *et al.*, 1996). Chinese water chesnut is a monocotyledon, and ferulic acid dimers have now been found in the cell walls of many plants in this category, partciluarly grasses (Ralph *et al.*, 1994). Most fruits and vegetables are dicotyledons and only *Beta vulgaris* (sugar beet and beetroot) conatin similar levels of ferulic acid to moncots (Ralet *et al.*, 1994). Recently low levels of ferulic acid have been detected in the cell walls of apple and bean pod (Waldron *et al.*, 1997).

1.4.2 Cell wall changes

Fruit softening in many fruits is associated with textural changes that are believed to result from disassembly of the primary cell wall. The process of degradation varies among species but it is thought that the solubilisation and depolymerisation of pectins and hemicelluloses are major events in softening. Redgwell *et al.* (1997) reported the softening characteristics of various temperate fruits. Fruits that ripened to a soft melting texture such as persimon, avocado, blackberry, strawberry and plum exhibited

pronounced wall swelling *in vitro*. However, fruits that had a firm, crisp texture when ripe, e.g. apple, nashi pear and watermelon, did not show cell wall swelling *in vitro*. The swelling of the cell wall was correlated with the degree of pectin solubilisation. It is now thought that the texture changes during ripening are more complex and involve other mechanisms that lead to pectin solubilisation and/or the breakdown of the cellulose-xyloglucan network. Batisse *et al.* (1996a) suggest that, the major difference between firm and soft cherries is the degree of polymerisation of pectin side chains. A high degree of polymerisation produces much more rigid cell walls, which could account for the more regularly shaped cells found in firm cherry fruits. Conversely, soft cherry fruits may have cells with a more irregular shape due to less pectic polymerisation.

In nectarines, solubilisation of pectin precedes depolymerisation and the loss of galactose although no increase in soluble galactose was recorded (Dawson *et al.*, 1992). In apricots it was reported that the decrease in pectin galactans and the reduction of pectin cross-linking within the pectin backbone was linked to the softening process during ripening (Femenia *et al.*, 1998). Redgwell and Harker (1995) suggested that the loss of cell wall associated galactose and pectin solubilisation in ripening kiwifruit are separate processes. Galactose loss may be necessary, but not sufficient for pectin solubilisation and fruit softening. Thus retention of cell wall galactose did not affect softening in kiwifruit (Redgwell and Harker, 1995). In ripening melon fruit, which soften over a 24 – 48 hour period, the initial pectin solubilisation coincided with the loss of galactose from the cell wall but preceded the increased expression of PG mRNA. (Rose *et al.*, 1998). Depolymerisation only occurred in the later ripening stages along

with the accumulation of PG mRNA. Depolymerisation of hemicelluloses, however, was detected at the onset and throughout ripening.

1.4.1.1 Breakdown of the cellulose-xyloglucan network

Ripening is not the only process in which the cell wall is modified. During cell expansion, the wall needs to loosen to allow growth to occur. Xyloglucan metabolism is likely to be highly co-ordinated during cell expansion, as a lack of xyloglucan synthesis would effect the integrity of the wall. The rate and extent of hemicellulose breakdown is fruit specific. A study of the ripening of Charentais melons indicated that one of the early events of softening was the selective degradation of a sub-population of xyloglucans that were tightly bound to cellulose (Rose *et al.*, 1998). O'Donoghue *et al.* (1997) found that modification of xyloglucan polymers occurred early in ripening pepino fruit and this coincided with an initial loss in firmness. During the later stages of ripening there were changes in the amount and molecular weight of chelate-soluble pectin. Further evidence of the changes in hemicellulose and pectin relating to softening was reported by Kojima *et al.* (1994) who suggested that the co-ordinated degradation of pectins, hemicellulose polysaccharides and starch is the main cause for the loss of firmness of banana pulp.

The role of cellulose breakdown is less clear because of the difficulties in studying intact microfibrils. Chemical treatments that are used to remove contamination of hemicelluloses from cellulose microfibrils may solubilise cellulose chains in the

paracrystalline regions (Rose and Bennett, 1999). Sakurai and Nevins (1997) reported that the net cellulose content of avocado mesocarp cell wall did not change during ripening. O'Donoghue *et al.* (1994) has reported a small downshift in the molecular weights of unbranched cell wall polymers, presumably cellulase.

1.4.2 Cell Wall Hydrolases

Many enzymes, that are capable of cell wall hydrolysis have been identified in fruit. The most common of these are cellulase or endo-β-(1-4) glucanase (EGase), polygalacturonase (PG), pectinmethylesterase (PME) and β-galactosidase.

1.4.2.1 Polygalacturonase

A wide range of plant enzymes are known to catalyse pectin modification and disassembly including exo- and endo-PGs, pectate lyase, PME and β-galactosidase. PG hydrolyses the α(1-4) link between adjacent demethylated galacturonic acid residues. PGs and PMEs may act synergistically in the breakdown of pectic polysaccharides. PG participates in many plant development processes (Hadfield and Bennett, 1998), but the majority of the research on this enzyme has been in relation to fruit ripening, mostly in tomato. The solubility of pectins in transgenic tomato fruit in which PG mRNA accumulation was suppressed by 99%, remained at wild-type levels but depolymerisation of solubilised pectins was suppressed (Smith *et al.*, 1990). Even in

tomato plants which were modified to inhibit the expression of PG to very low levels, the fruit still softened (Schuch *et al.*, 1991, Langley *et al.*, 1994).

The tomato mutant, *rin*, which does not ripen or soften and has no PG activity was transformed with a full length PG cDNA coupled to an ethylene induced promoter. The resultant transgenic fruit showed PG activity, pectin solubilisation and depolymerisation comparable to wild-type plants (Giovannoni *et al.*, 1989). The fruit did not soften and were not altered in any other way, suggesting that PG-mediated pectin disassembly is not by itself sufficient for normal fruit softening. However, transgenic fruits over expressing PG were less susceptible to pathogen attack than fruits with wild-type levels of PG activity (Kramer *et al.*, 1991). The evidence is that PG-mediated pectin degradation does not contribute to early fruit softening but contributes to tissue deterioration in the late stages of fruit ripening. There is little evidence that PGs play an important role in fruit softening in strawberry.

1.4.2.2 Pectinmethylesterase

PME is widely distributed in plants and fungi (Tove *et al.*, 1998). PME acts to remove the methylester groups in the C6-position of galacturonic acid and acts synergistically with PG to generate sites for PG action (Tucker, 1993). Different isoforms of PME have been reported and this has complicated the study of this enzyme in fruit. Gaffe *et al.* (1994) have reported that the majority of PME activity in ripening tomato fruit is due to three isoforms. These are of two types: (I) PME isoforms synthesised during fruit

ripening and (II) PME isoforms present throughout growth and development in all tissues (Gaffe *et al.*, 1994). Antisense experiments demonstrated that group I PME isoforms neither affect fruit development and ripening nor plant growth and development (Hall *et al.*, 1993). Reduced PME causes an almost complete loss of tissue integrity during fruit senescence but has little effect on fruit firmness (Tieman and Handa, 1994). A study of PME in fruit of the orange has shown the enzyme to be mainly localised in the outer layers of the juice vesicles, in the outer cell layers of the lamellae between the segments and in the outer layers of the albedo in the peel (Tove *et al.*, 1998). A study by Blumer *et al.* (2000) of tomato showed that PME was first detected adjacent to seeds in immature green fruit and was later detected only in tissue adjacent to the cuticle during ripening. The PME levels in the fresh-market cultivars were lower than those in the processing cultivar. The total detectable PME levels in the fresh-market fruits increased during ripening whereas the PME levels in the processing fruit peaked at the breaker stage (Blumer *et al.*, 2000).

1.4.2.3 β-galactosidase

Another enzyme which can break down the pectic polysaccharides is β-galactosidase. This enzyme has been detected in a wide range of fruits including tomato (Carey *et al.*, 1995), peach (Ju *et al.*, 2000), mango (Ketsa *et al.*, 1999), durian (Ketsa and Daengkanit, 1999), raspberry (Iannetta *et al.*, 1999), carambola (Chin *et al.*, 1999), pear (Perdue *et al.*, 1998) and grape (Barnavon *et al.*, 2000). The enzyme releases galactose from cell wall polymers and is ripening related (Ross *et al.*, 1993, Ross *et al.*, 1994). The release

of galactose has been correlated with an increase in β-galactosidase II activity in tomatoes during ripening (Carey *et al.*, 1995). A putative cDNA, *pTomβgal* 4, encoding β-galactosidase II was cloned from tomato fruit (Smith *et al.*, 1998). The *pTomβgal* 4 transcript was detected at the breaker stage and exhibited maximum expression at the turning stage before decreasing during the later stages of ripening. The transcript was present in all tissues of the fruit but highest levels occurred in the outer tissues. Perdue *et al.* (1998) reported the isolation of three isoforms of β-galactosidase that had different levels of activity during ripening in "d'Anjou" pears. Recently, seven β-galactosidase genes expressed during tomato fruit development have been identified (Smith and Gross, 2000). As with PME activity studies, assaying total activity of β-galactosidase does not give an accurate picture of its role in fruit ripening. In papaya, an increase in β-galactosidase activity correlates with pectin and hemicellulose modification (Lazan *et al.*, 1995).

1.4.2.4 Cellulase (endo 1,4-β-D-glucanase)

Increases in EGase activity during fruit ripening have been shown in (Pesis, 1978), strawberry (Abeles and Takeda, 1990), raspberry (Sexton *et al.*, 1997) and cherimoya (Sanchez *et al.*, 1998). Although there are temporal correlations between fruit ripening and increases in cellulase activity it still has to be shown that there is breakdown of native cellulose in fruits during ripening. It has been suggested that the *in vivo* substrate

for cellulose is xyloglucan. EGase hydrolyses β-1,4-linked glucans *in vitro* and increases in EGase activity correlate with a decrease in the average molecular size of xyloglucan in many fruits (Brummell *et al.*, 1994). Sexton *et al.* (1997) demonstrated that cellulase activity increases in the abscission zones in red raspberry (*Rubus ideaus* L. cv Glen Cova) fruit indicating a role in fruit separation.

EGase appears to be the most likely candidate for fruit softening in strawberry. Recently two different EGase cDNA clones have been isolated from strawberry fruit (Harpster *et al.*, 1998, Manning, 1998, Llop-Tous *et al.*, 1999, Trainotti *et al.*, 1999a, b). The first of these to be reported, FANR97 (Manning, 1998) but now named *cel*1 is fruit specific and is expressed in a ripening related manner under the control of auxin. This was also reported by Harpster *et al.* (1998) who showed that auxin applied to white deachened fruit repressed accumulation of cellulase mRNA. The second strawberry EGase, *cel*2 (Trainotti *et al.*, 1999b) is expressed in vegetative tissue and developing and ripening fruit. From the spatial and temporal expression, it appears that *cel*1 is more closely related to softening than *cel*2. Recently, *cel*1 which shows high homology to tomato *cel*2, was specifically down regulated in transgenic strawberry fruit. Although the *cel*1 transcript was undectectable in some transformants fruit firmness was not affected (Woolley *et al*, personal communication). Tomato *cel*2, one of seven EGases genes identified in tomato, shows expression in ripening tomato fruits and abscission zones (Brummell *et al.*, 1999a). The suppression of tomato *cel*2 does not affect fruit softening but does increase the force required to break abscission zones (Brummell *et al.*, 1999a). In tomato, *cel*2 has an important role in cell separation but its role in fruit softening is

unclear, as is the role of strawberry *cel*1. In strawberry it appears that *cel*2 plays a role in both fruit softening and cell wall expansion (Llop-Tous *et al.*, 1999, Trainotti *et al.*, 1999b)

Further evidence of synergistic activity between different EGase isoforms was reported by Bonghi *et al.* (1998) who observed different temporal expression of two EGase genes in ripening peach fruit. The pI 6.5 EGase was the only one present during the early growth stage and the pI 9.5 EGase was the most abundant during ripening. The role of EGase in the breakdown of cell walls during fruit ripening is still unclear with further research needed to identify the *in vivo* substrates of these enzymes and to study the effects of modifying their expression.

1.4.2.5 Pectate lyase

Pectate lyases are extensively characterised pathogen-secreted enzymes, which cleave β-1,4-linked galacturonosyl residues of pectins. Recently Medina-Escobar *et al.* (1997) have isolated a fruit specific pectate lyase clone which is expressed in ripening strawberry fruit under the control of auxin. A pectate lyase clone has also been isolated from banana, which is fruit specific and under hormonal control (Dominguez-Puigjaner *et al.*, 1997, Medina-Suarez, 1997).

1.4.2.6 Xyloglucan endotransglycosylase

Xyloglucan endotransglycosylase (XET) has been proposed to be involved in cell wall loosening (Nishitani and Tominaga, 1992). The mode of action of this enzyme is to cleave a xyloglucan backbone and graft one of the chains to the non-reducing end of a second xyloglucan chain (Cosgrove, 1999). Percy *et al.* (1996) reported that XET activity in apple and kiwifruit was highest two weeks after anthesis when cell division was highest. The activity of XET then decreased sharply in both fruits but increased again during fruit growth. In apple, XET activity peaked at harvest time and then decreased after harvest. XET activity in maturing kiwifruit had a similar profile to that in apple but the activity continued to increase after harvest until the fruit was ripe. In kiwifruit, the early increase in XET activity was in core tissue whereas in the mesocarp the enzyme only increased after harvest. In another study of XET expression in kiwifruit (Chen *et al.*, 1999), a very low level of XET mRNA was detected in the fruit at harvest time. It is an interesting posibility that the differences in XET activity between apple and kiwifruit may be related to the fact that ripe apple fruit have a crisp texture whereas ripe kiwifruit have a soft texture. Catala *et al.* (2000) reported that XET expression was highest during tomato fruit growth and that its expression profile was similar to that of the expansin (LeExp2) and endo-1,4-β-glucanase (Cel7) genes. No expression of these genes was detected during fruit ripening. Transgenic tobacco plants with reduced XET expression contain xyloglucan with increased molecular size compared to wild-type plants (Herbers *et al.*, 1998). Accumulating evidence indicates that XET has a role in cell wall assembly during cell division and expansion and although XET appears to play

no direct role it role in fruit softening it may prepare parts of the cell wall for disassembly.

1.5 EXPANSINS

Expansins are proteins which were first identified by McQueen-Mason *et al.* (1992) as mediators of pH-dependent cell wall extension and stress relaxation. Expansins do not have hydrolytic activity (McQueen-Mason and Cosgrove, 1995) and appear to act by disrupting the noncovalent bonding between cellulose and hemicellulose (McQueen-Mason and Cosgrove, 1994). Two families of expansins, α- and β-expansins, have now been recognised. The two types of expansin have about 25% amino acid identity to each other and they have very similar effects on cell wall rheology (Cosgrove, 1999).

The α-expansin family have a high degree of sequence similarity, with about 75% amino acid identity (Cosgrove, 1999). Much of the detailed work on this group of proteins has been carried out on cucumber hypocotyl walls. When α-expansins extracted from cucumber hypocotyl tissue were applied to excised *Arabidopsis* hypocotyls, they significantly increased elongation. This was also observed using tomato shoot meristems and tobacco suspension culture cells showing that cucumber hypocotyl α-expansins can affect tissues from different species (Cosgrove, 1999). Researchers have detected α-expansins in a range of expanding tissues, in rice internodes (Cho and Kende, 1997),

cotton fibres (Shimizu *et al.*, 1997), *Arabidopsis* leaves (Shcherban *et al.*, 1995), in strawberry fruit (Harrison *et al*, personal communication) (Civello *et al.*, 1999) and in tomato fruit (Rose *et al.*, 1997). Expansins are part of a large multigene family and so far over 20 have been identified in *Arabidopsis*, nine in tomato (Brummell *et al.*, 1999c), six in tobacco (Cosgrove, 1999) and six in strawberry (Harrison *et al*, personal communication).

In strawberry the six identified expansin cDNAs had unique temporal and spatial expression profiles during fruit development. Some expansins (FaExp3 and FaExp4) show an up-down-up profile, FaExp6 and FaExp7 show a down-up-down profile and FaExp5 and FaExp2 increase in expression during ripening (Harrison *et al*, personal communication). This supports the suggestion by Rose *et al* (1997) that some expansins have specialised roles during cell wall modification.

During tomato fruit development expansins *Exp3*, *Exp4*, *Exp5*, *Exp6* and *Exp7* were differentially expressed whereas only *Exp1* was present in high levels during fruit ripening (Brummell *et al.*, 1999c). This was also reported by (Rose *et al.*, 2000, Rose *et al.*, 1997) who showed that expansin LeExp1 was specifically expressed in ripening fruit under the regulation of ethylene. Tomato fruit in which the expression of expansin *Exp1* was suppressed to 3% of wild-type levels were firmer than control fruit (Brummell *et al.*, 1999b). Although the transgenic fruit were firmer, the depolymerisation of polyuronides was only inhibited late in ripening and the breakdown of hemicelluloses was not affected. Tomato fruits that overexpressed *Exp1* were softer than controls

(Brummell *et al.*, 1999b). The tomato fruit were softer at the mature green stage than the control fruit and this correlated with an extensive breakdown of the hemicellulose network. The depolymerisation of polyuronides was unaffected. Brummell *et al.* (1999b) suggested that *Exp1* is responsible for loosening of the cellulose-hemicellulose network prior to breakdown.

The β-expansin family have more divergent sequences than the α-expanisns (Cosgrove, 1999), but appear to be homologous in structure to the α-expansins (Cosgrove *et al.*, 1997). Of the β-expansins only the *Zea m*1 has been studied for its action on cell walls. *Zea m*1 is a group-1 grass pollen allergen and has similar rheological effects on cell walls as cucumber α-expansins but shows higher specificity towards monocot cell walls (Cosgrove *et al.*, 1997). Grasses also have α-expansins whereas only one β-expansin with homology to an *Arabidopsis* EST has been found in dicots.

It is very likely that expansins are a key element in fruit softening causing the loosening of polymers in the cell wall to allow cell wall hydrolytic enzymes access to their substrates. Further studies using sense and antisense technology to modify the expression of expanisns and other genes either singly or in different combinations will be required to elucidate the roles of these various cell wall proteins.

1.6 HORMONAL AND GENETIC CONTROL OF RIPENING

1.6.1 Ethylene

Fruit ripening is under the control of plant growth regulators. The growth regulators auxin, gibberellin and cytokinin generally inhibit ripening and ethylene and abscisic acid enhance ripening (Tucker, 1993). Climacteric and non-climacteric fruits can be distinguished by their patterns of ethylene synthesis during fruit ripening. Climacteric fruits including avocado and banana exhibit a burst of ethylene production with the onset of ripening. Non-climacteric fruits such as lemon and orange exhibit a decline in ethylene synthesis during ripening (Tucker, 1993). Ripening is not the only process in which ethylene is synthesised as wounding induces ethylene synthesis in both climacteric and non-climacteric fruits.

Adams and Yang (1979) established the ethylene biosynthetic pathway in apple (Fig 1.2) which is thought to be common in all higher plants. The two key enzymes in this biosynthetic pathway are 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. Hamilton *et al.* (1990) isolated pTOM13, a cDNA from ripe tomato that encoded ACC oxidase. Transgenic tomato plants in which pTOM13 was down regulated had suppressed levels of ACC oxidase mRNA and produced lower levels of ethylene during ripening and following mechanical wounding of leaves (Hamilton *et al.*, 1990). The cDNA was expressed in transformed yeast cells and these produced ethylene in the

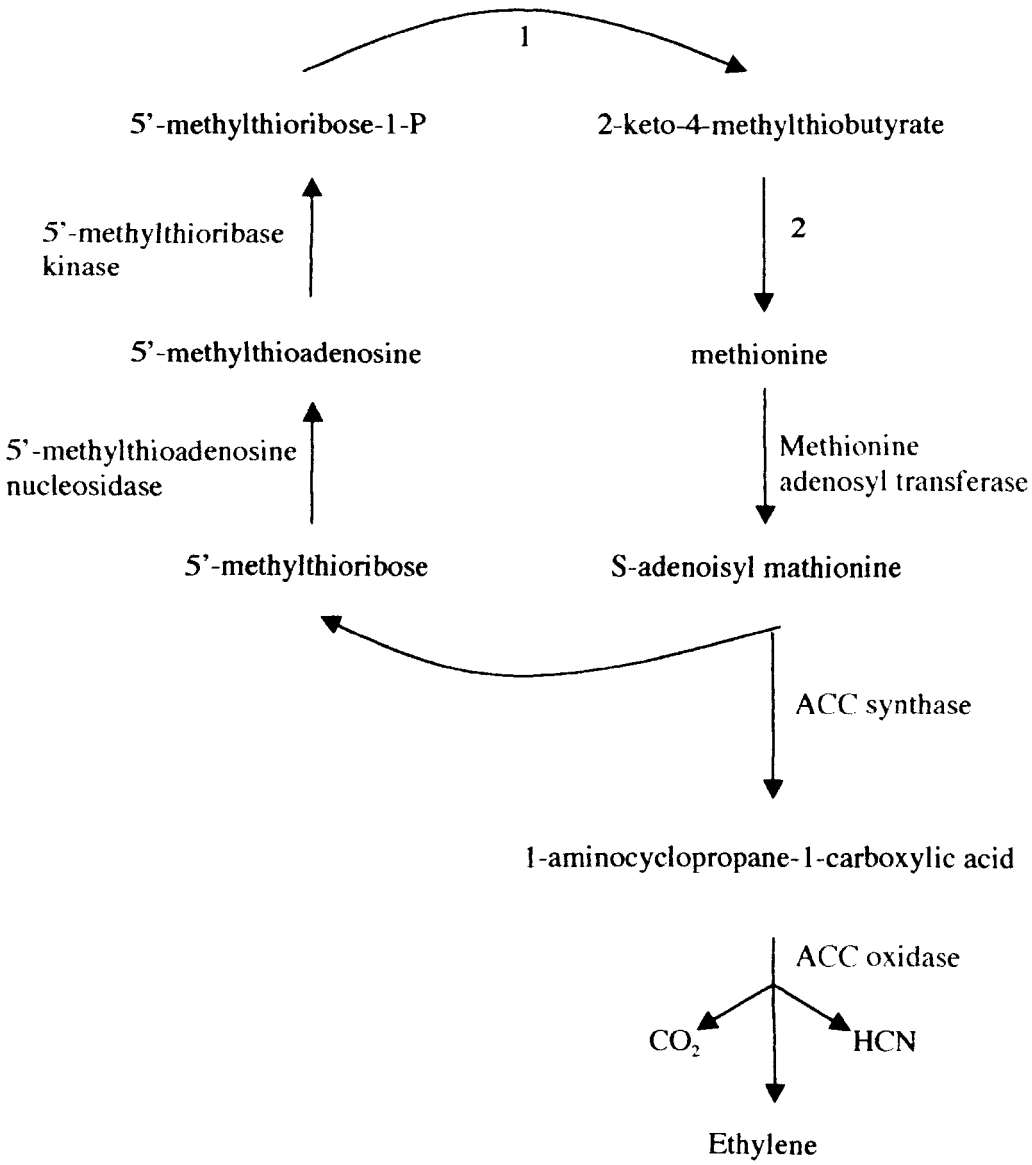

Figure 1.2 Ethylene biosynthetic pathway from Tucker (1993). 1: This step is catalysed by at least three enzymes. 2: This step represents a transamination reaction with glutamate as the most efficient amino donor.

presence of ACC confirming *pTOM*13 to be ACC oxidase (Hamilton *et al*., 1991). Fruits of Charentais melons plants expressing an antisense ACC oxidase transgene had much reduced levels of ethylene compared to control plants and their ripening was inhibited on and off the vine (Ayub *et al.*, 1996). Inhibition of ripening in these fruits was reversed by application of exogenous ethylene indicating that suppression of ACC oxidase does not affect ethylene perception. Although ripening was inhibited in the antisense plants, i.e. the fruit did not soften, there was no difference in the carotenoid content of the flesh between transformed and control fruit but chlorophyll loss was affected (Ayub *et al.*, 1996). These results show that in melon, fruit softening and chlorophyll loss are ethylene dependent processes and that carotenoid accumulation is independent of this gaseous hormone. During ripening of climacteric fruits, levels of ACC rise rapidly from the mature green stage to the ripe stage (Tucker, 1993). The levels of ACC remain high in the ripe fruit but ethylene production decreases, suggesting that ACC oxidase activity rather than ACC synthase is being regulated. In Japanese apricot, ACC synthase expression increased prior to the increase in expression of ACC oxidase (Mita *et al.*, 1999). Exposure of the mature green fruit to exogenous ethylene induced the expression of ACC synthase as well as ACC oxidase. In addition, wounding the fruit pericarp induced the expression of ACC synthase but not ACC oxidase (Mita *et al.*, 1999). This indicates that there are probably two control systems for ethylene production in plants. Differential expression of ACC synthase could have accounted for the differences in ethylene production in two melon varieties (Shiomi *et al.*, 1999). Perkins-Veazie (1995) reported no autocatalytic ethylene production in

strawberry, which is possibly due to low levels of ACC synthase in fruit before and during ripening.

1.6.1.1. Ethylene perception

Components of the ethylene signal transduction pathway (Fig 1.3) have been identified by characteriszation of ethylene-response mutants in *A. thatliana*. A number of putative ethylene receptors have recently been isolated from tomato (Tieman *et al.*, 2000, Tieman and Klee, 1999, Payton *et al.*, 1996, Wilkinson *et al.*, 1995b) and muskmelon (Sato-Nara *et al.*, 1999) which show homology to the *Arabidopsis* ETR1 ethylene receptor. Transgenic tomato plants that have reduced LeETR4 expression are very sensitive to ethylene and show enhanced flower senescence and accelerated fruit ripening (Tieman *et al.*, 2000). It is thought that LeETR4 is a negative regulator of ethylene response in tomato plants. Further work is still needed to deduce the roles of different ethylene receptors and other components of the signal transduction pathways that mediate the response from perception to gene expression.

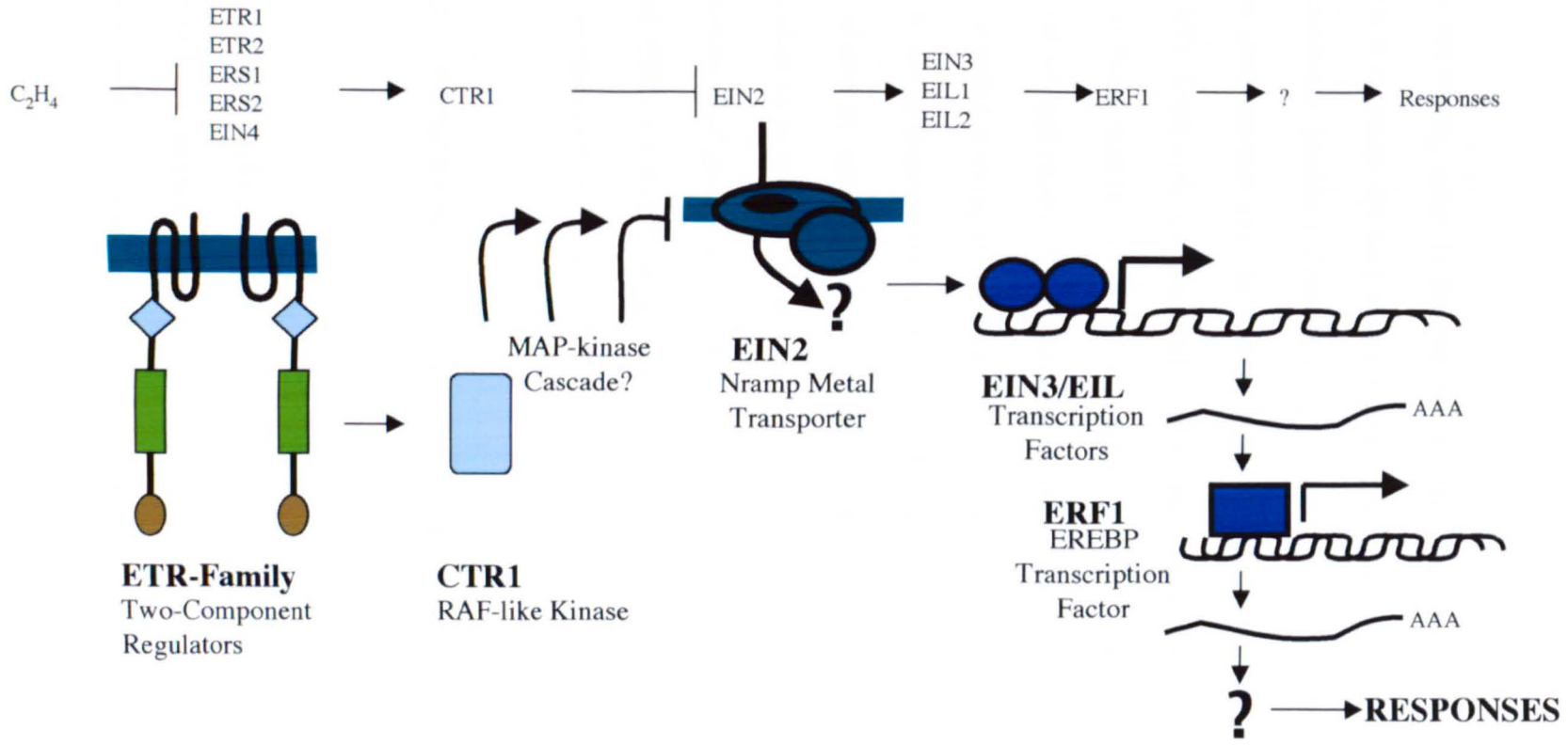

Figure 1.3 The genetic interactions and biochemical identities of components of the ethylene signal transduction pathway from Bleecker and Kende (2000). Ethylene is thought to regulate negatively a family of membranes-associated receptors that are related to the bacterial two-component superfamily of catalytic receptors. The histidine-kinase transmitter domains of members of the receptor family interact with the regulatory domain of the Raf-like kinase CTR1. This receptor/CTR1 complex negatively regulates a membrane protein (EIN2) related to a superfamily of metal transporters. The cytoplasmic C-terminal domain of EIN2 positively signals downstream to the EIN3 family of transcription factors located in the nucleus. A target of the EIN3 transcription factors is the promoter of the ERF1 gene, a member of a second family of transcription factors. ERF1 is rapidly induced in response to ethylene and is capable of activating a subset of ethylene responses when ectopically expressed.

1.6.2 Auxin

In strawberry, auxin is the major hormone affecting fruit ripening. Unlike ethylene, which enhances ripening in climacteric fruits, auxin acts as a repressor of ripening in strawberry. Studies indicate that specific auxin binding in strawberry fruit membranes are proteinaceous and show specificity for certain auxin analogues (Naryanan *et al.*, 1981). Southwick and Poovaiah (1987) demonstrated that polar auxin movement in strawberry receptacles appears to promote uniform growth at some distance from the point of application. The removal of achenes from one half of a mature green fruit accelerated ripening in the de-achened half as indicated by anthocyanin accumulation and loss of firmness (Given *et al.*, 1988a). Given *et al* (1988) hypothesised that auxin produced by achenes inhibits ripening in green fruit and that as fruit develop auxin production by the achenes decreases modulating ripening in the receptacle. Evidence for the control of gene expression during ripening by auxin was provided by Manning (1994 and 1998) in which the expression of many *in vitro* translated polypeptides and cDNAs were repressed by auxin.

1.7 GENE EXPRESSION DURING RIPENING

The complex physiological and biochemical changes that occur during fruit ripening result from changes in the activity of enzymes or other proteins. The levels of many of these proteins correlate with gene expression indicating that ripening is under

transcriptional control. As previously described the changes in gene expression are modulated by plant hormone regulators such as ethylene or auxin. Many ripening related genes have been discovered by analysing mRNA changes during fruit development and amongst the proteins they encode are cell wall degrading enzymes, ethylene biosynthetic enzymes and enzymes involved in pigment biosynthesis.

Strawberry fruit has been used as a model for studying ripening in non-climacteric fruit (Nam *et al.*, 1999, Manning, 1998, Medina-Escobar *et al*, 1997, Wilkinson *et al.*, 1995a). Nam *et al* (1999) isolated eight ripening-induced cDNAs from wild strawberry by differentially screening a cDNA library. None of the clones appeared to encode functions identifiable with ripening metabolism. Manning (1998) identified 26 families of ripening related cDNAs from a differential screen between mature green and ripe strawberry fruit. Many of the cDNAs identified in this study encode proteins likely to be involved in key metabolic events during ripening including protein turnover (cysteine protease), flavour (ester), biosynthesis (pyruvate decarboxylase), translational activity (elongation factor 2), auxin conjugation (UDP-glucose IAA-glucosyl transferase) and sucrose transport Manning (1998). Both Nam *et al* (1999) and Manning (1998) have identified a cDNA from strawberry fruit that encodes for acyl carrier protein (ACP). Manning (1998) hypothesised that as there is no increase in lipid content in ripening strawberry fruit, ACP is involved in the production of volatile flavour compounds many of which are likely to be derived from lipid biosynthesis.

Wilkinson *et al.* (1995a) used the PCR-differential display to identify five ripening enhanced cDNAs. One of these, encoding chalcone synthase (CHS), could be linked to anthocyanin biosymthesis a key metabolic event in ripening strawberry fruit. CHS catalyses the key enzymatic step in flavanoid biosynthesis, i.e., the conversion of 4-coumaroyl-CoA from the general phenylpropanoid pathway into narigenin chalcone (Hahlbrook and Scheel, 1989). The partial sequence of this clone was very similar to one of the clones isolated by Manning (1998). The expression of CHS parallels the accumulation of anthocyanins in ripening strawberry fruit (Manning, 1998, Wilkinson *et al.*, 1995a).

Other researchers have investigated gene expression during ripening in banana (Clendennen and May, 1997, Medina-Suarez, 1997), raspberry (Jones *et al.*, 1998) and blackcurrant (Woodhead *et al.*, 1998). What is common is that genes are expressed in fruits encoding proteins associated with key metabolic events and secondary metabolism.

1.8 AIMS OF PROJECT

The aim of this project was to use the technique of bulked segregant analysis (Michelmore, 1991) to identify the genetic determinants of fruit texture in strawberry. To obtain an F_1 generation segregating for firmness a cross was made between a strawberry cultivar producing firm fruit (ITA 80-52-1) and a cultivar with soft fruit

(Tamella). Fruits from the F_1 plants will be analysed quantitatively for the firmness phenotype (Chapter 3). Two pools will be produced from plants with the firmest and softest fruits. In theory, pooling of firm and soft fruit should equalise cDNAs unrelated to texture thereby minimising differences in genetic background enabling genes specifically realted to texture to be identified. The two pools will be analysed by the recent molecular techniques of suppressive subtractive hybridisation (Chapter 4) and cDNA-AFLP (Chapter 5). The expression of interesting cDNAs obtained from these screens will be analysed in the two pools to examine their relationship to fruit softening.

Chapter 2: MATERIALS AND METHODS

2.1 PLANT MATERIAL

Pollen from *Fragaria ananassa* cv. ITA 80-52-1 was transferred to emasculated flowers of *F. ananassa* cv. Tamella and 446 seedlings were obtained. The seedlings were allowd to become dormant naturally and then vernalised at 2°C for 6 weeks in a cold store to induce flower initiation. The plants were forced into flowering by moving into a greenhouse with 16hr light period and 20°C day and 12°C night temperature regime. Flowers were pollinated every other day using a paintbrush.

2.1.1 Fruit Sampling

Fruits were selected at the mature green and ripe stages of development. The mature green stage was defined by the achenes being fully developed, starting to separate and set into the receptacle tissue that retained a hint of green. The mature ripe was defined by the fruit having an overall bright red colour, i.e. the ideal eating stage. The fruits were sampled three times a day, early morning, late morning and mid afternoon, to ensure they were at an equivalent stage of ripeness. After firmness measurements, the fruit were frozen in liquid nitrogen and stored at -70°C for mRNA extraction. Two fruits at each stage of development were sampled from each plant and duplicate firmness measurements made on every fruit. The dimensions of each fruit were recorded by measuring the maximum diameter across two perpendicular planes and its length.

2.2 MEASUREMENT OF FRUIT FIRMNESS

Fruits were sliced in half longitudinally and for each half, a thin slice containing the skin and achenes was removed parallel with the longitudinal cut. The firmness measurement was taken on this exposed part of the fruit flesh using a Stevens Penetrometer with a probe diameter of 4mm travelling at 50mm/sec. The force applied as the probe was pushed into the fruit was monitored on a chart recorder adjusted to have a full-scale deflection (fsd) of 2V for mature green fruit and 0.5V for ripe fruit. The position of inflection (the point of tissue failure) was converted into Newtons on the basis that 10.9N = 1V. This value was determined using a digital voltmeter during load cell calibration. The load cell was calibrated by hanging a weight (1kg) from the load cell and adjusting (as necessary) the reading on the voltmeter using the load cell trimmer.

2.3 EXTRACTION OF TOTAL RNA FROM FRUIT

2.3.1 Large scale extraction of RNA

The method used for RNA extraction was adjusted from Manning (1991). RNA was extracted from 10g of de-achened pooled fruit in 25ml of extraction buffer (0.2M boric acid, 10mM Na_2EDTA, pH7.6) in the presence of 0.5mls 25% sodium dodecyl sulfate (SDS) and 0.5ml 2-mercaptoethanol. The extract was brought to room temperature and

35ml of the lower phase of a phenol/chloroform mixture was added and mixed thoroughly. The mixture was centrifuged at 20,000g for 10 min and the upper aqueous phase was decanted and retained. The lower phase and inter-phase were re-extracted with 10ml of extraction buffer and recentrifuged. The upper phase was decanted and combined with the previous upper-phase and adjusted to 50ml with sterile water. 1.4 vol of sterile water and 0.1 volumes of 1M sodium acetate/acetic acid (pH 4.5) were added and thoroughly mixed. To precipitate polysaccharides 0.4 volumes of 2-butoxyethanol (2-BE) were added to this mixture, thoroughly mixed and incubated on ice for 30 minutes. The mixture was spun at 20,000g for 10 minutes at 4°C and the supernatant was decanted and retained. The supernatant containing total nucleic acids was mixed with 0.6 volumes, with respect to the volume before the addition of 2-BE, of 2-BE and incubated on ice for 30 minutes before centrifugation at 20,000g for 10 minutes. The pellet was washed consecutively with 40mM sodium acetate/acetic acid (pH4.5):2-BE (1:1v/v), 70% (v/v) ethanol and 100% ethanol and dried *in vacuo*. The pellet was dissolved in 1ml of autoclaved water and 500μl of 12M LiCl were added and mixed before incubating on ice for at least 1 hour. The precipitated total RNA was pelleted by spinning at 20,000g at 4°C for 10 minutes. The pellet was washed consecutively with 3M LiCl, 70% (v/v) ethanol, and 100% ethanol and dried *in vacuo*.

2.3.2 Microscale isolation of total RNA from strawberry fruit.

The microscale isolation method is a modification of the large scale method. Only 1g of ground tissue is used and all solution additions are one tenth of the large scale method.

2.3.3 Standard precipitation of DNA and RNA

RNA and DNA were precipitated with 2.5 vol ethanol and 2 vol ethanol respectively in the presence of 0.1M NaÂc/Hac pH 6.0 or 0.8 vol isopropanol. The mixtures were incubated for 30 minutes on ice and centrifuged at 13000 rpm in a microfuge at 4°C for 20 min. The pellet was washed consecutively with 70% (v/v) ethanol and 100% ethanol and dried *in vacuo*.

2.4 ANALYSIS OF GENE EXPRESSION BY *IN VITRO* TRANSLATION

2.4.1 In vitro translation of RNA

Total RNA (10μg) was added to a reaction mix of 4μl translation mix (Amersham, UK), 2μl 2.5M K acetate, 1μl 25mM MgÂc, 4μl ^{35}S-methionine (>1000Ci mmol-1) (Amersham, UK) and 39μl sterile distilled water. The reaction was incubated at 30°C for two hours after which 5μl of RNAse A (1mg ml^{-1}) was added and the reaction was incubated at 30°C for a further 15 minutes. The completed reaction was stored at –20°C.

2.4.2 Determination of radioactivity incorporated into *in vitro* translation products

Duplicate 2μl aliquots of translation mixture were diluted in 187.5μl of sterile water and added to 312.5μl of freshly prepared decolorising solution, consisting of a mixture of 6% hydrogen peroxide and 1M sodium hydroxide containing 1mM L-methionine (1:4v/v). The mixture was incubated at room temperature for 10 minutes. A 5μl aliquot from each duplicate was removed and added to 5ml of scintillant to determine total radioactivity. The remainder was added to 500μl of ice cold 25% (w/v) TCA, mixed thoroughly and incubated on ice for 10 minutes. The mixture of precipitated proteins was vacuum filtered onto 25mm diameter Whatman GF/C filters. The filters were washed consecutively with 4 x 5ml 5% TCA (2 x 5ml used to wash the tubes), 1 x 10ml 5% TCA and 1 x 20ml 100% ethanol to reduce unincorporated radioactivity. The filters were dried in an oven at 60°C, then placed in scintillation vials with 5ml of scintillant for counting.

2.4.3 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2.4.3.1 First dimensional isoelectric focusing

The ^{35}S-labelled translated polypeptides were separated by 2D-PAGE using "Immobiline® DryStrip" (IPG) (Pharmacia, UK) for the first dimension. The IPG strips were first rehydrated by soaking in rehydration buffer of 8M urea, 0.5% (w/v) (CHAPS),

15mM DTT and 0.2% (w/v) Pharmalyte (pH 3-10), overnight. The ^{35}S-labelled polypeptides (55μl) were concentrated by precipitating with four volumes of acetone and incubating on ice for 10 minutes and dissolved with 20μl sterile distilled water. The mixtures were spun at 13,000 rpm for 5 minutes and the supernatant removed. The pellet was washed with 500μl of acetone and respun. The supernatant was decanted and the pellet was air-dried for 15 minutes. The dried pellet was dissolved in 20μl of fresh lysis buffer containing 9.5M urea, 2% (w/v) CHAPS, 0.8% Pharmalyte (pH 3-10) and 1% (w/v) DTT. The samples were loaded onto the rehydrayted IPG strips using silicone rubber frames as wells placed 2.5 cm from the anode. The IPG strips were run at 150V for 30 minutes, then 300V for 1 hour and finally 1200V for 16 hours.

2.4.3.2 Second dimension SDS-PAGE

The IPG strips were equilibriated in 2x 10ml washes of 6M urea, 30% glycerol, 2% SDS, 5mM Tris-HCl (pH 8.45) and a trace of bromophenol blue for 15 minutes for each wash. Separating gels were prepared with 10% (w/v) total acrylamide (T) and 0.1% (w/v) NN' methylenebisacrylamide (C) in a buffer of 1M Tris-HCl, pH 8.45. A 25mm stacking gel (4%(w/v) acrylamde) was prepared and added to the top of the separating gel. Prior to transfer, the IPG strips were cut to a size of 160mm by removing 10mm from the anode and cathode ends. The IPG strip was loaded onto the stacking gel and covered with 0.5% (w/v) agarose. The gel was run with an anode buffer of 0.2M Tris, pH 8.9 and a cathode buffer of 0.1M Tris, 0.1M Tricine, 0.1% (w/v) SDS pH 8.25. An

initial voltage of 60V was used and after two hours this was increased to 80V until the dye front was 1cm from the bottom of the gel. The gel was fixed in isopropanol:water:acetic acid (25:65:10) for 30 minutes and then soaked in the fluor Amplify™ (Amersham, UK) for a further 30 minutes. The gel was dried under vacuum at 80°C and exposed to X-ray film (BioMax, Kodak, UK) for 26 days at -80°C.

2.5 ISOLATION OF POLY (A)$^+$ RNA FROM TOTAL RNA

Poly (A)$^+$ RNA was isolated from total RNA by affinity chromatography on oligo dT cellulose by a method adapted from Bantle *et al.* (1976). Total RNA was denatured at 65°C for15 minutes and diluted with an equal volume of 2x loading buffer (1.0M LiCl, 0.2% (w/v) SDS, 40mM Tris/HCl (pH 7.7), 2mM Na_2EDTA). Columns were packed with 100mg of oligo dT cellulose which had been washed several times with sterile water to remove fine particles. The column was equilibrated in x1 loading buffer and the denatured RNA applied to the column. The eluate was collected, denatured again and reapplied to the column. The column was washed with 4 x 1ml loading buffer and the washings were combined and retained ("high salt"). Bound mRNA was eluted from the column with 4 x 1ml low salt buffer (10mM Tris/HCl (pH 7.5), 1mM Na_2EDTA, 0.5% (w/v) SDS). The low salt eluates were combined and the mRNA was precipitated in the presence 1μl glycogen (20mg/ml) as carrier and 0.1M NaAc/Ac (pH 6.0) by 0.8 vol isopropanol. The pellet was washed with 1x1ml 70% (v/v) ethanol, 1x1ml 100% EtOH, dried and dissolved in denaturing buffer [10μl Tris/HCl (pH 7.7), 90μl DMSO, and 10μl of LiCl buffer (1.0M LiCL, 0,05mM Na_2EDTA, 2.0% SDS, 10mM Tris/HCl (pH 6.5)].

The sample was denatured at 55°C for 10 minutes, combined with 0.9ml of 1x loading buffer and added to the column. After re-applying the eluate to the column and washing with 4x 1ml loading buffer the mRNA was eluted with 4x 1ml "low salt" buffer as before. The "high salt" and "low salt" elutes were each precipitated with 0.8 vols. of isopropanol, with glycogen being added to the "low salt" buffer to aid precipitation. The "high salt" pellet was dissolved in 500μl of sterile distilled water and 10μl removed for UV analysis. The "low salt" pellet was dissolved in 500μl of autoclaved distilled water and all of the sample taken for UV analysis in cuvettes previously washed with DEPC. Following UV determination, the mRNA was reprecipitated by ethanol.

2.6 cDNA SUBTRACTION

Poly $(A)^+$ RNA prepared from pools of the firm and soft fruits was used for suppressive subtractive hybridisation using the PCR-Select™ cDNA Subtraction Kit (Clontech) (See chapter 4, Figure 4.1).

2.6.1 Driver Preparation

Driver double stranded DNA was synthesised from poly $(A)^+$ RNA using the Amersham cDNA synthesis module. The cDNA synthesis was performed in two parallel reactions, one using 1.5μg poly $(A)^+$ for the main synthesis and the other using 0.5μg poly $(A)^+$ for the tracer reaction, monitored by the incorporation of ^{32}P. The newly synthesised cDNA

was phenol extracted, precipitated and stored under 100% ethanol at -20°C. The labelled cDNA samples were air dried and dissolved in 30μl of distilled H_2O. The samples were electrophresed on a 1% agarose gel under alkaline conditions (50mM NaOH). The gel was fixed with by incubating 2x 30 minutes in 7% (w/v) TCA. The gel was dried and exposed to film for 6 hours. The unlabelled cDNA was digested by *Rsa*I at 37°C in a 50μl reaction containing 15 units of *Rsa*I. Aliquots were removed after 1 hour and 1.5 hours for gel analysis. The digestion was terminated by the addition of an EDTA/glycogen mix. The digested cDNA was phenol extracted, precipitated and dissolved in 5.5μl of distilled water.

2.6.2 Tester Preparation

*Rsa*I digested driver (1.5μl) was diluted with 7.5μl sterile water. Adapters 1 and 2 (2μl, 10μM) (Table 2.1) were incubated with 2μl of diluted cDNA and 4μl master mix [3μl autoclaved water, 2μl 5X ligation buffer (20mM Tris-HCl (pH 7.8), 50mM $MgCl_2$,

Table 2.1 Sequences of the PCR-Select cDNA PCR adapters

Adapter 1
5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5'
Adapter 2
5'-TGTAGCGTGAAGACGACAAGAAAGGGCGTGGTGCGGAGGGCGGT-3' 3'-GCCTCCCGCCA-5'

Table 2.2 Sequences of the PCR-Select cDNA PCR primers

Primer	Sequence
PCR Primer	CTAATACGACTCACTATAGGGC
Nested PCR Primer (NP1)	TCGAGCGGCCGCCCGGGCAGGT
PCR Primer	TGTAGCGTGAAGACGACAAGAA
Nested PCR Primer (NP2)	AGGGCGTGGTGCGGAGGGCGGT

10mM DTT and 0.25 mg ml^{-1} bovine serum albumin)] in separate reactions over night at 16°C. The reactions were terminated by the addition of 1μl of EDTA/glycogen mix (0.2M EDTA; 1 mg ml^{-1} glycogen). The reactions were incubated at 72°C for 5 minutes to inactivate the ligase. A third control ligation, which acts as a positive control for ligation and a negative control for subtraction, was prepared containing both adapters.

2.6.3 Subtractive Hybridisation

One and a half microlitres of driver cDNA was added to each of two tubes containing 1.5μl of either adapter 1 or adapter 2 ligated tester cDNA and 1.0μl of hybridisation buffer. The solution was overlaid with one drop of mineral oil, the cDNA was denatured (100°C for 20 seconds) and allowed to reanneal at 68°C for 8 hours. After the first hybridisation, the two reactions were mixed together in the presence of diluted denatured driver (1.0μl) and allowed to hybridise at 68°C for 20 hours. The mixture was diluted with 200μl of dilution buffer (20mM HEPES-HCl (pH 8.3), 50mM NaCl, 0.2mM EDTA (pH8.0), heated at 75°C for 7 minutes and stored at -20°C.

2.6.4 PCR Amplification

The primary PCR was performed in a total volume of 25μl, containing 1μl diluted subtracted cDNA, 1μl PCR primer P1 (10μM), 1μl PCR primer P2 (10μM) (Table 2.2), 2.5μl 10x PCR reaction buffer, 0.5μl dNTP (10μM), 0.5μl 50x Advantage cDNA polymerase mix (Clontech), 18.5μl sterile water). Primary PCR was performed under the

following conditions: 34 cycles of 94°C for 30 sec, 68°C for 30 sec, 72°C for 1.5 minutes. The amplified product was diluted ten-fold and 1μl of diluted product was used as the template for the secondary PCR. The same conditions were used as for the primary PCR except that only 12 cycles performed and that the PCR primers P1 and P2 were replaced by nested PCR primer 1 (NP1) and nested PCR primer 2 (NP2) (Table 2.2). The PCR products (5μl) were analysed on a 2% (w/v) agarose gel.

2.7 CLONING SUBTRACTED cDNAS INTO pPCR-SCRIPT AMP SK(+)

The PCR products were purified through a sepharose gel matrix (G50, Pharmacia, UK) spin column. The purified PCR products (10μl) were blunt ended by adding 1μl 10mM dNTP mix (2.5mM each), 1.3μl 10x polishing buffer and 1μl cloned Pfu DNA polymerase (0.5U) (Stratagene), overlaying with one drop of mineral oil and incubating for 30 minutes at 72°C. The polished products (4μl) were ligated into 10ng of the pPCR-Script cloning vector (Stratagene) in a reaction containing, 1μl 10x reaction buffer, 0.5μl 10mM rATP, 5U *Srf*I enzyme 4U of T4 DNA ligase made up to 10μl with sterile distilled water. The reaction was incubated at room temperature for 1 hour and then terminated by incubating at 65°C for 10 minutes. For the transformation aliquots of 40μl of XL10-Gold Kan (Stratagene) ultracompetent cells were transferred to 15ml Falcon 2059 tubes on ice. The cells were incubated with 1.6μl of 1.42M β-mercaptoethanol for 10 minutes on ice, before adding 2μl of the cloning reaction. After incubating on ice for 30 minutes, the cells were heat pulsed at 42°C for 30 seconds and then incubated on ice for 2 minutes. NZY$^+$ broth (0.45ml), preheated to 42°C, was added to the transformation

reaction and incubated for 1 hour on a shaker at 37°C. The transformation reaction (50μl) and 50μl NZY$^+$ broth were spread on the LB plates containing carbenicillin (50μl ml^{-1}), 2% X-gal and 10mM IPTG and incubated at 37°C overnight.

2.8 DIFFERENTIAL SCREENING OF THE SUBTRACTED cDNA CLONES

To detect inserts, colonies were analysed by PCR. White colonies were picked and suspended in 25μl of autoclaved water. A 1μl aliquot of resuspended cells was added to 9μl of autoclaved water. The mixture was overlaid with one drop of mineral oil and incubated at 95°C for 5 minutes. An aliquot of 10μl of master mix [0.25μl DYNAZYME (2U l^{-1}) (Genzyme), 2.0μl x10 PCR buffer (Genzyme), 0.05μl 20mM dNTP mixture (Pharmacia), 0.4μl M13 forward primer (10pmol μl-1) (Sigma), 0.4μl M13 reverse primer (10pmol μl-1) (Sigma), 6.9μl autoclaved water] was added. The PCR was performed with the following parameters: one cycle of 96°C for 30 seconds, 55°C for 1 minutes and 72°C for 2 minutes, 34 cycles of 96°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes and one cycle of 72°C for 10 minutes. The PCR products (10μl) were analysed on a 2% (w/v) agarose gel. PCR products from single colonies that contained inserts were denatured by adding 10μl of PCR products with 10μl 0.5M NaOH, incubated at room temp for 5 minutes and 2μl dot-blotted onto duplicate nylon filters. The subtracted cDNA probes were digested with *Rsa*I as described earlier. The digested probe (200ng) was labelled with ^{32}P-dATP using the HYPER-Prime™ Random Primed DNA labelling kit (Bioline, UK). The digested probe was diluted 1:1 and 1μl was incubated at 100°C for 5 minutes with 2μl of primer mix and allowed to cool to room

temperature. The primer/probe mix was incubated at 37°C for 30 minutes with 3μl dNTP mix (Low C mix), 2μl x10 reaction buffer, 5μl (α-^{32}P) dCTP (Amersham, UK), 6μl autoclaved water and 1μl DNA polymerase. The labelling reaction was terminated by incubating at 65°C for 10 minutes. Filters were prehybridised with 0.25M phosphate buffer and 7% (w/v) SDS at 65°C overnight. Denatured cDNA probes were hybridised to blots using 0.25M phosphate buffer and 7% (w/v) SDS at 65°C. The filters were washed twice with 2x SSC, 1% (w/v) SDS for 15 minutes, and once with 0.2x SSC, 1% (w/v) SDS for 15 minutes. Excess solution was removed from the blots with blotting paper, covered with Saran wrap and exposed to X-ray film (BioMax, Kodak) at –70°C for two weeks.

2.9 NORTHERN ANALYSIS

Total RNA was isolated from firm both parents and soft fruit pools. RNA (10μg of total RNA per lane) was dissolved at 1μg μl^{-1} in loading buffer [2.2M formaldehyde, 50% formamide, 5% (w/v) glycerol, 50μg ml^{-1} ethidium bronide, 20mM MOPS acid, 5mM sodium acetate, 1mM EDTA, 0.01% bromopnenol blue]. The RNA samples were denatured for 15 minutes at 65°C and separated on a 1.4% agarose/0.22M folmaldehyde denaturing gels with an electrode buffer containing 0.22M formaldehyde. The RNA was transferred to positively charged membranes (Boehringer) by capillary blotting and fixed by UV light for 5 minutes. Transcripts were detected with ^{32}P-labelled probes as described above. The filters were prehybridised, hybridised and washed using similar conditions to the dot-blot filters.

2.9.1 "Virtual northern" analysis

"Virtual northerns" were prepared by using the ligated cDNA in section 2.10.1 as template. The template was amplified using the *Eco*RI and *Mse*I core primers and the same reaction conditions as the primary AFLP amplification and using the following parameters: a denaturation step at 94°C for 30s an annealing step at 56°C for 1 minute and an extension step for 12 minutes at 72°C for 25 cycles of amplification. The amplified cDNA (10μl) was separated on a 2% (w/v) agarose gel until the dye front had moved 1cm. The cDNA was transferred to positively charged membranes (Boehringer) by capillary blotting and fixed by UV light for 5 minutes. Transcripts were detected with ^{32}P-labelled probes as described above. The filters were prehybridised, hybridised and washed using similar conditions to the dot-blot filters.

2.10 AFLP FINGERPRINTING

2.10.1 Template preparation for PCR

Templates were prepared from cDNA synthesised from poly $(A)^+$ RNA isolated from the firm and soft pools and restricted by digestion with a combination of *Eco*RI and *Mse*I. cDNA (300ng) was incubated at 37°C with 5U of *Eco*RI and 5U of *Mse*I in 40μl containing x1 SureCut buffer (Promega). After digestion, 10μl of a ligation mixture containing 5pMol *Eco*RI-adapters, 50 pMol *Mse*I-adapters (Table 2.3), 1U T4 DNA

ligase and buffers were added and incubated at 14°C overnight. After ligation the reaction was diluted to 500μl with 10mM Tris-HCl, 0.1mM EDTA pH 8.0 and stored at -20°C.

2.10.2 PCR amplification

For the pre-amplification, *EcoR*I-T and *Mse*I-C primers (Table 2.4) were used. Mixtures contained 5μl of the diluted primary template, 30ng *EcoR*I-T primer, 30ng *Mse*I-C, 1U BioTaq (Bioline, UK), 1.0μl buffer containing 1.5mM MgCl2 and 0.2mM of all four dNTPs. The amplifications were carried out on a PE-9600 thermocycler as follows, 94°C denaturation for 30s; 56°C annealing for 30s and 72°C polymerisation for 1 min for 20 cycles. The primer *EcoR*I-TTA was end-labelled using 10U T4 polynucleotide kinase and 100μCi [γ^{33}P] ATP. The labelling reaction was performed in 50μl 25mM Tris-HCl (pH 7.5), 10mM MgCl2, 5mM DTT, 0.5mM spermidine-3KCl using 500ng oligonucleotide primer, 100μCi [γ^{33}P] ATP and 10U T4 polynucleotide kinase. The reactions (10μl) contained 5ng labelled *EcoR*I-TTA primer (0.5μl from labelling reaction), 30ng *Mse*I-CTT primer, 2.5μl template from the pre-amplification, 1U BioTaq, 1.0μl buffer containing 1.5mM MgCl2 and 0.2mM of all four dNTPs. Thermocycling was as follows: a denaturation step at 94°C for 30s an annealing step (see below) for 1 minute, an extension step for 12 minutes at 72°C. The annealing temperature was

Table 2.3 Sequences of the cDNA-AFLP adapters

Eco R1 adapter	*Mse* 1 adapter
5-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5	5-GACGATGAGTCCTGAG TACTCAGGACTCAT-5

Table 2.4 Primer sequences used for the primary and secondary PCR amplifications of the cDNA-AFLP.

Primer	CORE	ENZ	EXTENSION
Eco RI primary	GACTGCGTACC	AATTC	T
Mse I primary	GATGAGTCCTGAG	TAA	C
Eco RI secondary	GACTGCGTACC	AATTC	TTA
Mse I secondary	GATGAGTCCTGAG	TAA	CTT

reduced from 65° for the first cycle to 56°C in 0.7°C steps for 11 cycles and subsequently maintained at 56°C for 23 cycles.

2.10.3 Analysis of PCR products

The samples were heated at 90°C for 5 minutes after the addition of 15μl of loading buffer (98% formamide, 10mM EDTA pH 8.0 bromophenol blue and xylene blue as tracking dyes) and quickly cooled on ice. Each sample (5μl) was loaded onto a 6% polyacrylamide sequencing gel (Sequagel®-6, National Diagnostics, UK). A 1x TBE buffer (100mM Tris/100mM Boric acid/2mM EDTA) was used as running buffer. Elecrophoresis was at constant power, 35W, until the xylene blue dye front had moved half way down the gel. The gel was transferred to 3mm paper (Whatman), dried under vacuum at 70°C and then exposed to x-ray (BioMax, Kodak) film at -80°C overnight after a few drops of the AFLP reaction were used to mark the dried gel for orientation purposes.

2.10.4 Isolation of AFLP fragment

The X-ray film and dried gel were aligned and the AFLP bands of interest carefully cut out of the gel with a clean scalpel blade. The cDNA fragment was eluted from the dried gel by incubating in elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA (pH 8.0), 0.1% (w/v) SDS) at 37°C for 16 hours. The sample was

centrifuged at 12,000g for 1 minute at 4°C and the supernatant was transferred to a fresh microfuge tube. An additional 0.5 volumes of elution buffer was added to the pellet, vortexed and recentrifuged. The supernatant was combined with the previous supernatant and cleaned further by centrifuging through a sepharose gel matrix (G50, Pharmacia, UK). The cDNA was precipitated by adding 2 volumes of 100% ethanol and incubating on ice for 30 minutes. The cDNA was recovered by centrifuging at 20,000g for 10 minutes at 4°C and the pellet was washed consecutively with 70% and 100% ethanol and dried *in vacuo*. The dried pellet was dissolved in 200μl of sterile distilled water and reprecipitated with 2 volumes of 100% ethanol and 25μl of 3M sodium acetate (pH 5.2). The cDNA was recovered as before and dissolved in 10μl of sterile distilled water. The cDNA fragments were reamplified using the same PCR reaction as the secondary AFLP amplification, using the unlabelled AFLP primers *EcoRI*-TTA and *Mse*I-CTT and the following conditions: 35 cycles of 94°C for 1 minute, 56°C for 30 seconds and 72°C for 1 minute and 10μl of the PCR products were analysed on a 2% (w/v) agarose gel. The reamplified AFLP fragments were cloned into pCR-Script Amp SK(+) and transformed into Epicurian Coli XL1-Blue MRF' (Stratagene) or TOP10 One Shot™ (Invitrogen, Netherlands) cells. Colonies containing inserts were analysed by PCR using AFLP primers *EcoR*I-TTA and *Mse*I-CTT and the same conditions used to select clones for the differential screen. The PCR products were analysed on a 2% (w/v) agarose gel. AFLP fragments which had *EcoR*I and *Mse*I ends were selected for DNA sequencing.

2.11 ZERO BLUNT™ TOPO™ CLONING OF PCR PRODUCTS

A 5μl cloning reaction was set up with 4μl of PCR product and 1μl of pCR®-Blunt II-TOPO vector (Invitrogen) and incubated at room temperature for exactly 5 minutes. The reaction was terminated by adding 1μl 6x TOPO™ Cloning Stop Solution, mixing thoroughly and then storing the terminated reaction on ice. One vial of TOPO One Shot™ per reaction was thawed on ice and 2μl of cloning reaction was added an the mixture incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42°C and immediately transferred to ice for 2 minutes. SOC medium was brought up to room temperature and 250μl added to the transformation reaction and incubated on a shaker for 1 hour at 37°C. LB plates containing 50μg ml^{-1} of kanamycin were prepared and 50μl of transformed cells were spread on the plates and incubated at 37°C overnight.

2.12 SYNTHESIS OF RNA PROBES, HYBRIDISATION AND MEMBRANE WASHING

RNA probe synthesis reactions were performed in a total volume of 20μl (2.0μl DNA template, 2μl 10X transcription buffer, 1μl 10mM ATP, 1μl 2mM modified CTP, 1μl 10mM GTP, 2μl 25μM UTP, 3μl labelled UTP, 2μl T3, T7 or SP6 RNA polymerase and distilled water to 20μl). The reaction was incubated at 37°C for one hour after which 1μl of DNAseI was added and incubated for a further 15 minutes. The labelled probe was added directly to the hybridising solution (0.25M phosphate buffer and 7% (w/v) SDS)

at 65°C overnight. Filters were prehybridised and washed as described earlier (section 2.8).

2.13 DNA SEQUENCING OF PCR PRODUCTS

DNA sequencing templates are generated by M13 forward and reverse primers to amplify an insert from the pCR-Script Amp SK(+) or pCR®-Blunt II-TOPO vectors using conditions used for detecting colonies with inserts. The PCR product was analysed on 2% (w/v) agarose gel to ascertain it was a single band. The PCR product was purified by centrifuging the PCR reaction through a sepharose gel column (G50, Pharmacia, U.K.). The purified product (1μl) was used as template with 1μl (3.2pmol μl^{-1}) of primer (M13 forward), 4μl of either BigDye mix or AmpliTaq FS and 5μl autoclaved water using the following profile in a DNA Engine (MJ Research, USA) thermal cycler as follows: 30 cycles of 96°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes. After cycle sequencing 2μl of 3M sodium acetate (pH 5.2) and 50μl of 95% ethanol was added to the reaction and incubated at room temperature for exactly 10 minutes. The mixtures were centrifuged at 20,000g for 25 minutes at room temperature and the pellet washed consecutively with 70% (w/v) and 100% ethanol and dried at 90°C for 2 minutes in a thermal cycler block. The dried sequencing reaction was either sequenced at Horticulture Research International using an ABI 377 automatic sequencer (Perkin-Elmer, UK) for BigDye reactions or at Durham University for AmpliTAq FS reactions.

Chapter 3: FRUIT FIRMNESS EVALUATION OF THE F_1 GENERATION

3.1 INTRODUCTION

In soft fruits, such as strawberry, texture is a key attribute affecting marketability. Texture is determined by the skin properties and flesh firmness and fruits with tougher skin and firmer fruit better tolerate handling and transportation and have a longer shelf life, thus improving their marketability. The penetrometer is a commonly used device to measure fruit firmness. It has a cylindrical probe with either a flat or concave tip. The probe is driven into the fruit tissue for a set distance and the maximum applied force is recorded. Various methods of testing fruit firmness using penetrometers have been developed with manual and automatic equipment (Harker, 1996). A motorised device was considered to be the best method as there was less variability in the measurements since the accuracy is not dependent on the operator.

The variation in a quantitative or qualitative trait within a segregating population can form the basis for identifying the genetic determinant of that trait. A method, bulked segregant analysis, which can identify specific markers in a genome has been developed by Michelmore *et al.* (1991). The method compares two pooled DNA samples from individuals in a segregating population sharing a common phenotype or genotype. The pooling strategy is to group common individuals sharing a specific trait so that the trait can be studied within a randomised genetic background. This method has been used to identify markers in lettuce, linked to a gene for resistance to downy mildew

(Michelmore *et al.* 1991); markers linked to a locus involved in Tomato Yellow Leaf Curl Virus (TYLCV) resistance (Chagué *et al.* 1997); a marker linked to a scab-resistance gene, V_f, in apple (Yang *et al.* 1997) and markers linked to the resistance of *Melamspora larici-populina* in *Populus* (Cervera *et al.* 1996). Yang *et al.* (1997) did not use a segregating population but used resistant cultivars and susceptible cultivars to produce the two bulked pools.

To obtain a population of strawberry plants segregating for fruit firmness a cross was made between a plant which produced very firm fruit (cv ITA 80-52-1) and a plant which produced very soft fruit (cv Tamella). The resulting F_1 generation was screened for fruit firmness to identify plants having the softest and firmest fruits for producing bulked pools of soft and firm fruits. In this way pooling would tend to equalise genetic differences not related to fruit texture thereby allowing the comparison of genes more specifically associated with this trait. Combined with the use of molecular techniques, it was hoped that this method should allow the identification of genes which determine fruit texture.

3.2 RESULTS

3.2.1 Sampling the F_1 population for firmness

Fruits at the mature green and ripe stage were harvested three times a day, in early morning, early afternoon and late afternoon. The firmness and dimension measurements were taken during a three hour period. From the initial 446 plants in the F_1 generation, fruit was only harvested from 269 plants, the others either failing to flower or succumbing to crown rot and mildew infection. For firmness measurements the fruit were sliced longitudinally and a thin layer of skin and achenes was removed by making a second cut parallel to the first, (Figure 3.1). This ensured that the fruit was stable and that all firmness measurements would be based on the inner-flesh and were not affected by the resistance of the skin. The firmness values were measured using a Stevens penetrometer in which a probe was pushed into the flesh at a fixed speed over a fixed distance. The force applied was continuously recorded and the point of inflection on the chart recorder (Figure 3.2) was taken to be the peak force (N) at the point of tissue failure. Plants of the F_1 generation were grown in one glasshouse compartment and placed randomly. The plant position was recorded and factored into the statistical analysis. The dimensions of the fruit and plant position were recorded to determine if these were related to firmness. The data were analysed in collaboration with the Biometrics Department at Horticulture Research International using Genstat 5 (release 3) software (see Appendix I for analysis program).

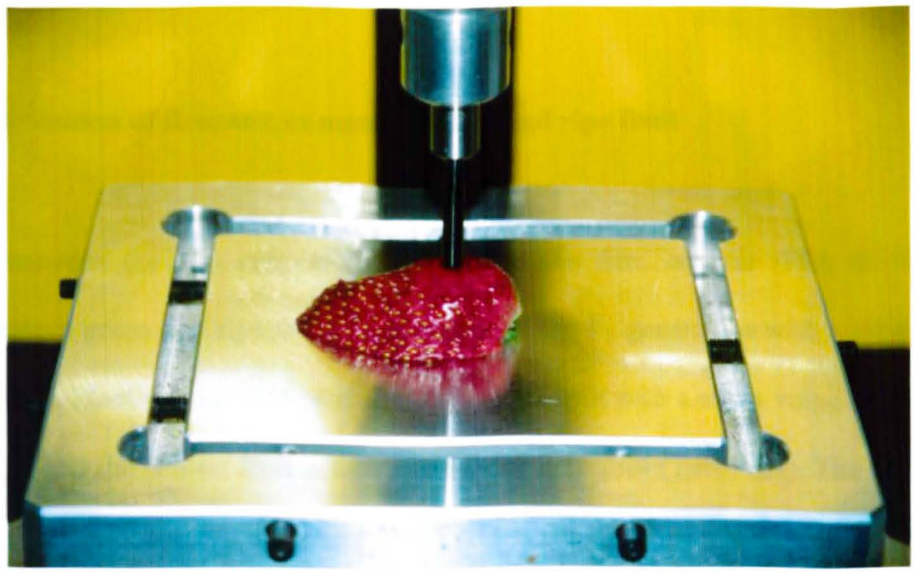

Figure 3.1 Prepared strawberry fruit on the penetrometer platform before measurement.

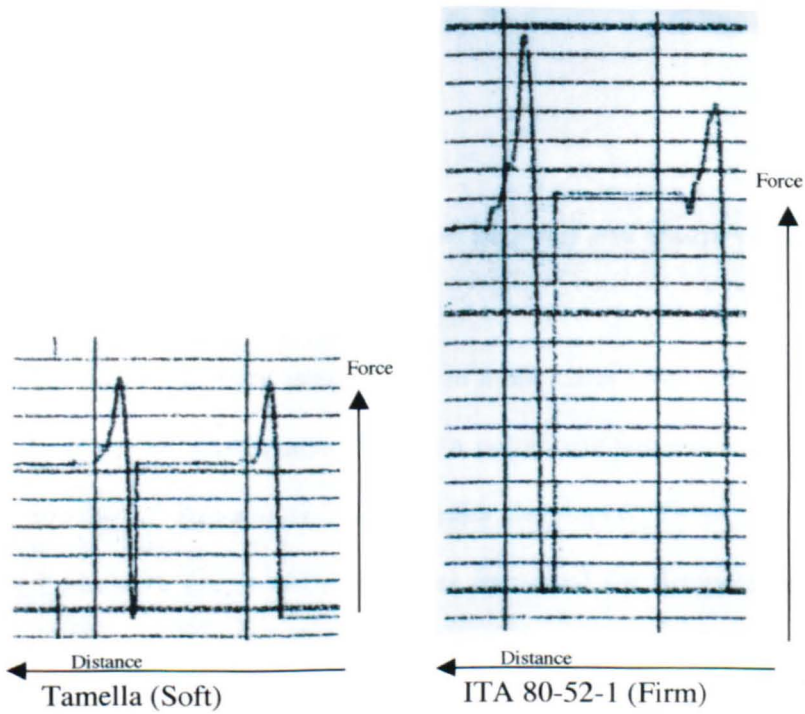

Figure 3.2 The firmness of fruits was measured by using a penetrometer. The force applied to the fruit flesh increases to a maximum and then suddenly decreases. The maximum force applied, just before tissue failure is used as a measure of firmness. Two recordings for each fruit are made. The mean firmness for Tamella fruit shown was 0.46N and for ITA 80-52-1 fruit 1.02N.

3.2.2 Distribution of firmness in mature green and ripe fruit

The cross between the soft cultivar (Tamella) and the firm cultivar (ITA 80-52-1) produced mature green and ripe fruits from plants of the F_1 generation with a range of firmness values. Plant number 324 produced the softest fruit with a mean value of 0.25N and plant 227 had the firmest fruit with a mean value of 1.23N (Table 3.1). The firmest fruit was therefore almost 5-fold firmer that the softest fruit. Mature green fruit showed a much larger difference, approximately 30-fold, between the softest and firmest fruit. Figures 3.3 and 3.4 show the spread of firmness values for mature green and ripe fruit, respectively. The unripe fruit histogram (Figure 3.5) shows that the firmness values are skewed towards softer fruit.

3.2.3. Relationship between fruit firmness values between and within plants

For some plants only ripe fruits were sampled and in a few cases only one ripe fruit was available. As pollination was done manually with a paintbrush some achenes were not fertilised and a number of abnormally shaped fruits developed. To ensure sampling consistency only fruits with a uniform shape were harvested for firmness analysis. If only a few fruit were produced by a plant then only ripe fruit were harvested. This accounts for many of the missing values for mature green fruit. The firmness of mature green fruit was recorded to test the hypothesis that it was related to the firmness of ripe

Table 3.1 The firmness and size index of the twenty softest and twenty firmest ripe fruits from the F_1 generation of the cross between cv ITA 80-52-1 and Tamella. The firmness was measured as the maximum force (N) exerted by the probe and was calculated as the mean value from the two halves of a fruit. See Appendices II and III for full data. The mean dimension (mm) was calculated by adding the three dimension measurements and dividing by three. See Appendices IV and V for full data

Firm Fruit			Soft Fruit		
Plant	Firmness (N)	Mean Dimension (mm)	Plant	Firmness (N)	Mean Dimension (mm)
227	1.228	28	324	0.253	32
19	1.078	24	31	0.260	41
45	1.021	24	360	0.260	28
292	0.989	25	269	0.273	23
189	0.962	21	109	0.280	31
364	0.942	28	309	0.307	33
111	0.941	37	308	0.307	31
288	0.935	30	37	0.307	31
334	0.920	33	328	0.328	27
258	0.908	31	368	0.335	33
74	0.902	26	130	0.335	28
397	0.901	30	72	0.341	29
253	0.901	34	86	0.348	22
132	0.900	29	46	0.355	37
394	0.895	24	255	0.362	31
350	0.893	26	123	0.362	26
121	0.888	22	384	0.362	31
347	0.860	29	231	0.369	32
203	0.853	26	148	0.369	34
411	0.847	24	427	0.375	33
Mean	0.938	28	Mean	0.324	31

Mean firmness for all F1 fruit 0.59N. Standard deviation 0.16N

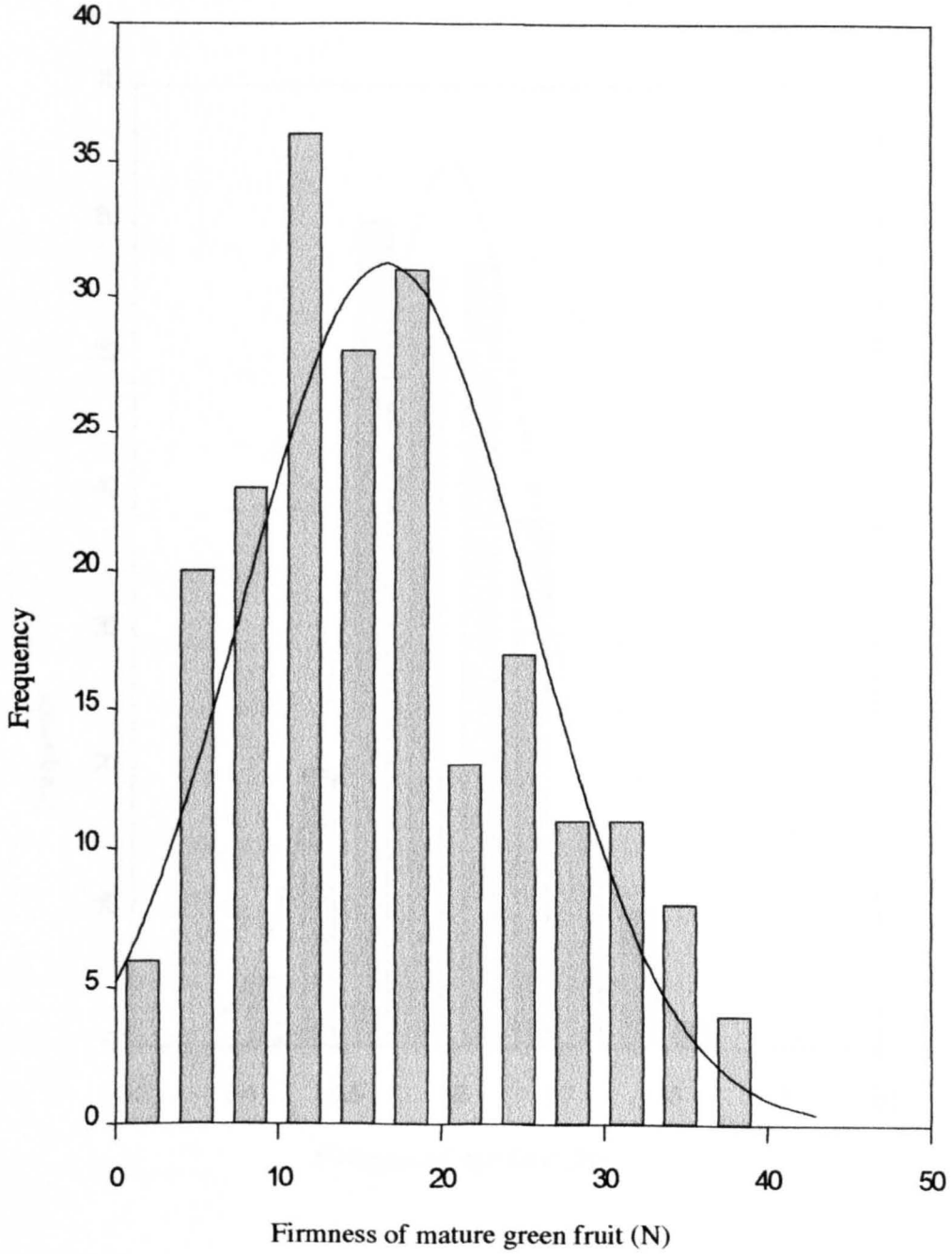

Figure 3.3 Distribution of the firmness of mature green strawberry fruit in the F_1 population of the cv ITA 80-52-1 x Tamella cross. The gaussian distribution was calculated using the sample mean and standard distribution of the population and superimposed on the bar chart.

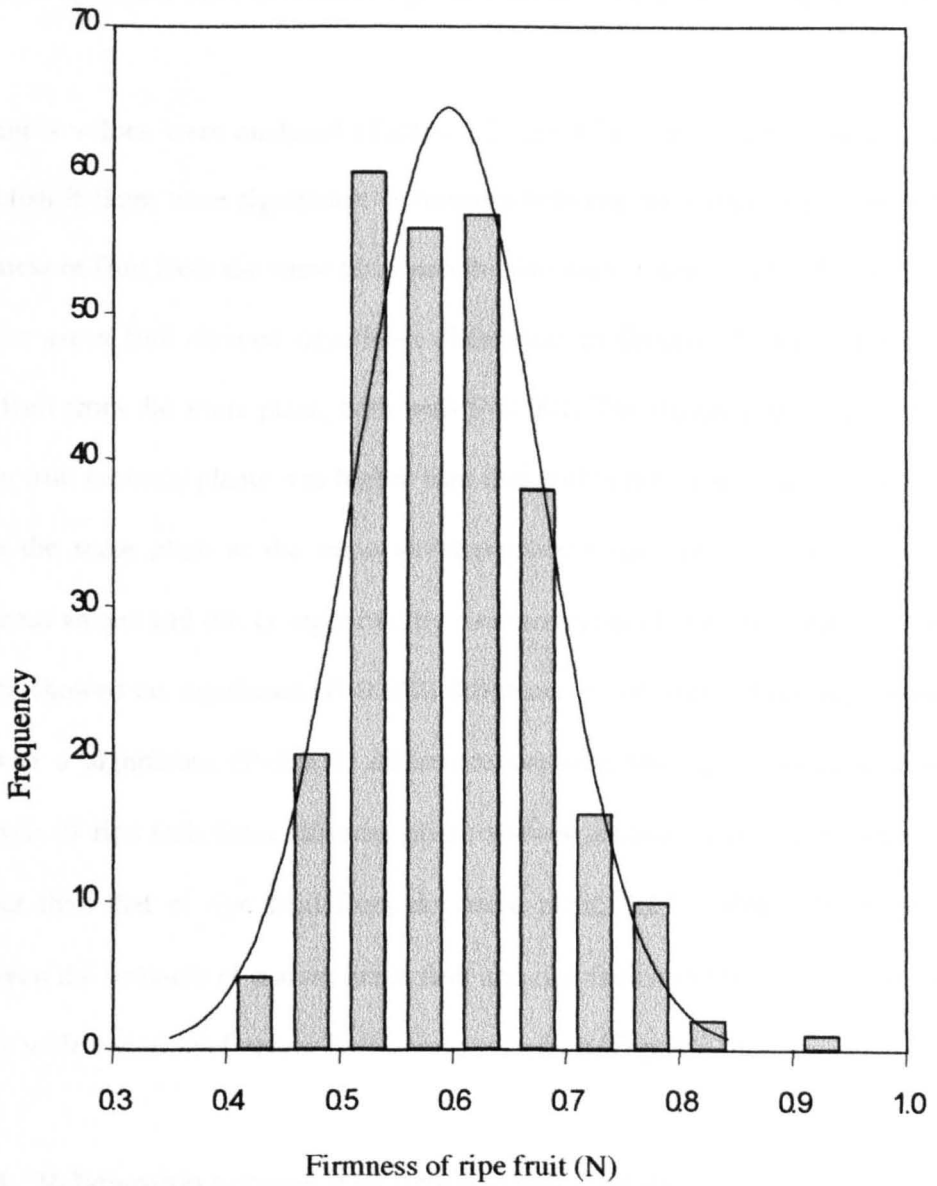

Figure 3.4 Distribution of the firmness of ripe strawberry fruit in the F_1 population of the cv ITA 80-52-1 x Tamella cross. The gaussian distribution was calculated using the sample mean and standard distribution of the population and superimposed on the bar chart.

fruit from the same plant, since it might be predicted that the texture of ripe fruit depends upon how the cell walls are synthesised and laid down during development.

Firmness values were analysed (Tables 3.2 and 3.3) with fruit size as a covariate to establish if there were significant differences between the firmness of fruit halves, the firmness of fruit from the same plant and the firmness of fruit from different plants. The mature green fruit showed significant differences in firmness between different plants and fruit from the same plant, both with $P<0.001$. The variance in firmness of mature green fruit between plants was higher than that within the same plant (Table 3.2). Fruits from the same plant at the same developmental stage are expected to have similar firmness values and this is supported by measurements of ripe fruit from the same plant which showed no significant ($P=0.262$) difference in firmness. Ripe fruit firmness only showed a significant ($P<0.001$) difference between fruit from different plants. The analysis of ripe fruit from different plants showed a variance of 15.26, which is much higher than that of ripe fruit from the same plant, 1.27 (Table 3.3). No correlation between the firmness of mature green fruit and ripe fruit was found. This may be related to the wide variation of values for mature green fruit (Figure 3.3).

3.2.4 Relationship between fruit firmness and fruit size

The relationship between fruit firmness and fruit size was analysed statistically. To take account of differences in fruit shape, fruit size was determined by measuring two perpendicular widths and the length of each fruit. This was converted into a size index

Table 3.2 Results of the analysis of variance for mature green fruit firmness with fruit size as a covariate. The correlation between fruit size and fruit firmness is shown.

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance	Covariance efficiency	F probability
Plants						
covariate	1	4136	4136	14.08		<0.001
residual	204	59921	293	2.94	1.06	
Fruits						
covariate	1	2749	2749	27.49		<0.001
residual	140	14004	100	15.58	1.19	
Fruit Samples	340	2182	6.42			

	coefficient
Plants	-0.88

Table 3.3 Results of the analysis of variance for ripe fruit firmness with fruit size as a covariate. The correlation between fruit size and fruit firmness is shown.

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance	Covariance efficiency	F probability
Plants						
covariate	1.00	1.55	1.55	15.26		<.001
residual	267	27.14	0.102	3.04	1.05	
Fruits						
covariate	1.00	0.042	0.042	1.27		0.262
residual	220	7.36	0.033	4.09	1.00	
Fruit Samples	489	4	0.008			

	coefficient
Plants	-0.0094

by calculating the mean dimension for the fruit i.e. adding the three dimension measurements and dividing by three. For ripe fruit there was a slight negative correlation, (-0.0094) between fruit size and firmness, i.e. larger fruit tended to be softer (Figure 3.5). However, this should be viewed against the considerable variation that occurs in ripe fruit (Table 3.3). The relationship was further investigated by comparing fruit from the twenty softest and firmest plants. In the twenty plants with the softest fruit (Figure 3.6) there was no clear correlation with size. Plants that produced the twenty firmest fruits did exhibit a slight negative trend but this was not considered significant as the gradient of the trend line was very small (-0.0004).

3.2.5 Pooling strategy

From the statistical analysis, plants with the twenty softest and twenty firmest ripe fruit were selected from the population (Table 3.1). The mean firmness of ripe fruit from the F_1 population was 0.59N with a standard deviation of 0.16. The mean firmness of the fruits in the firm pool was 0.324N with a standard deviation of 1.6 from the mean for the whole F_1 population. The mean firmness of the fruits in the soft pool was 0.937N with a standard deviation of 2.2 from that of all F_1 fruits. Wang and Paterson (1994) suggest that QTL alleles with phenotypic effects of 0.75 - 1.0 standard deviation or larger should be detectable in a population of manageable size (100 - 200 plant/lines). The use of phenotypically based pools should be able to identify QTL's having a very large effect. Sub-pools consisting of plants with the five and the ten softest and firmest ripe fruits were also made. With the pools of twenty plants there was a five-fold difference

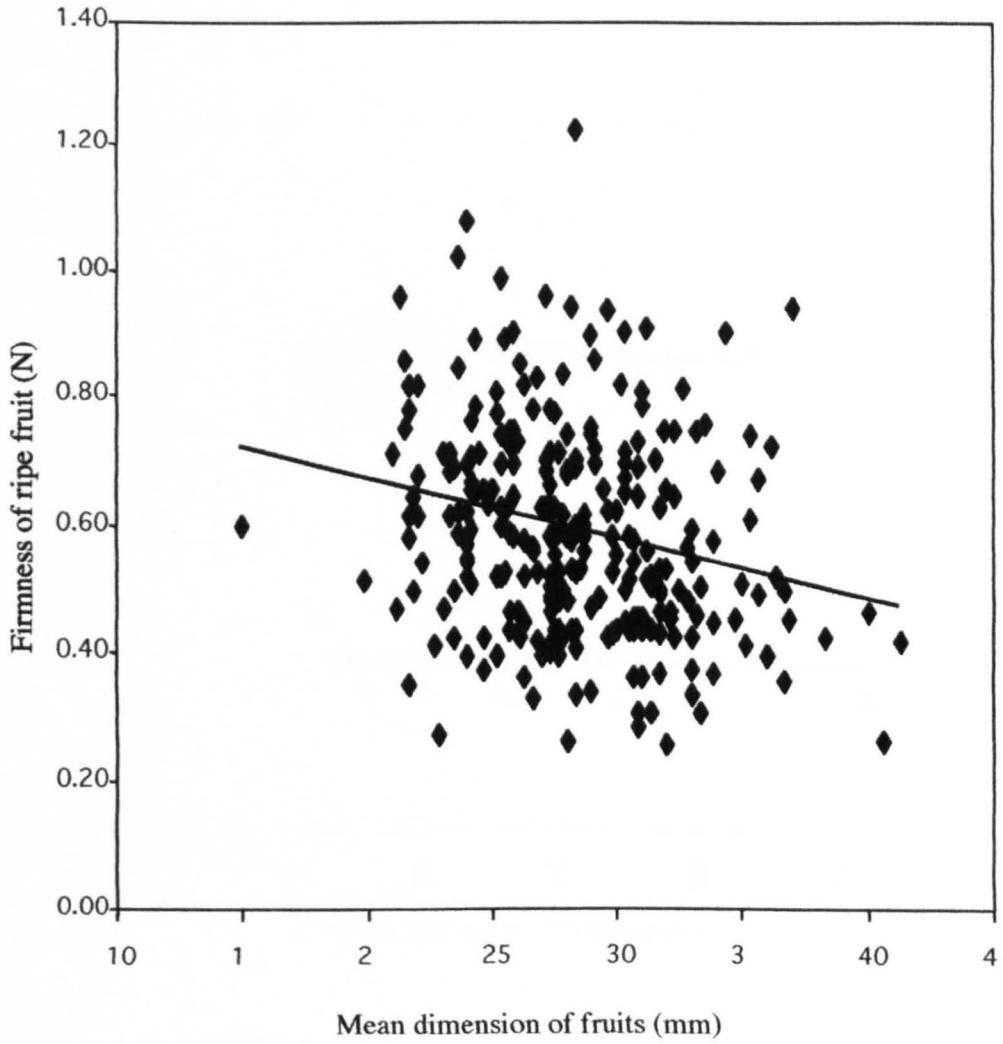

Figure 3.5 The relationship between size and firmness of ripe fruit within the F_1 generation. The mean dimension is calculated by adding the maximum distance across the fruit measured along three perpendicular axes and dividing by three. The gradient of the trend line is -0.0094.

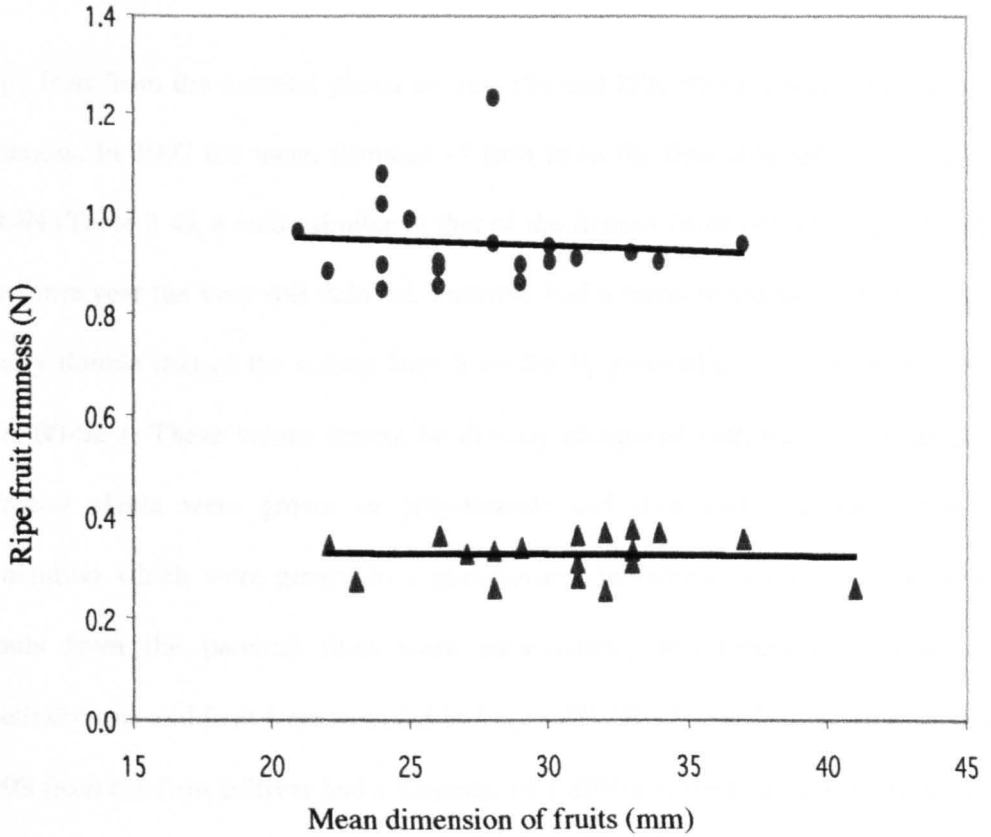

Figure 3.6 The relationship between fruit size and the firmness of the twenty softest (▲) and twenty firmest (●) plants. The mean dimension is calculated by adding the maximum distance across the fruit measured along three perpendicular axes and dividing by three.

between the softest and firmest fruits and a three-fold difference between the least soft and least firm fruits.

3.2.6 Firmness of fruits from strawberry parental lines

Ripe fruit from the parental plants cv Tamella and ITA 80-52-1 were also assessed for firmness. In 1997 the mean firmness of fruit from the firm cultivar, ITA 80-52-1, was 1.14N (Table 3.4), a value similar to that of the firmest fruits from the F_1 generation. In the same year the very soft cultivar, Tamella, had a mean firmness of 0.45N which was nearly double that of the softest fruit from the F_1 generation and less than half that of ITA 80-52-1. These values cannot be directly compared with the F_1 generation as the parental plants were grown in poly-tunnels and produced fruit later than the F_1 generation which were grown in a glasshouse and forced to flower early. The same plants from the parental lines were subsequently transferred to a glasshouse in Wellesbourne and fruit were sampled in May 1998. The fruits that were sampled in May 1998 from the firm cultivar had a firmness of 1.89N and from the soft cultivar the fruits had a mean firmness of 0.84N (Table 3.4). The soft cultivar, Tamella, remained less than half as firm as cultivar, ITA 80-52-1.

Table 3.4 Firmness of ripe fruit from cv Tamella (three plants) and ITA 80-52-1 (two plants). Fruits were analysed from plants grown in a poly-tunnel at East Malling (May 1997) and plants grown in a glasshouse at Wellesbourne (May 1998).

Year	1997		Year	1998	
	Tamella Firmness (N)	ITA 80-52-1 Firmness (N)		Tamella Firmness (N)	ITA 80-52-1 Firmness (N)
Fruit 1	0.35	1.23	Fruit 1	0.6	1.7
	0.6	1.28		0.65	1.8
Fruit 2	0.46	0.95	Fruit 2	0.84	1.17
	0.46	1.09		0.74	1.25
Fruit 3	0.38		Fruit 3	1.14	2.48
	0.27			1.09	2.94
Fruit 4	0.46				
	0.63				
Mean	0.45	1.14	Mean	0.84	1.89

3.3 DISCUSSION

The selection of fruits from the F_1 generation that differed in firmness depended on the penetrometer distinguishing one fruit from another. The penetrometer was able to identify a range of firmness in the population from 0.253N to 1.228N. Another important consideration in screening the population for firmness was to ensure all the fruits were at the same developmental stage. Ripe fruit were harvested on the basis of the red anthocyanin colour and mature green fruit were selected by comparing chlorophyll pigmentation and achene development and spacing. The colour of ripe strawberries is a continuously variable trait and the most important and convenient character when determining the stage of ripeness. Sacks and Shaw (1994) showed that for external colour traits the within fruit variance was greater than the among fruit variance and that taking two samples from several fruit replicates was an efficient way of characterising a genotype's fruit colour. The results of this screen have shown that selecting fruits by visual inspection is valid as there is much lower variance between fruits from different plants than amongst fruits from the same plant (Table 3.3). This is not the case when selecting the mature green fruit because the variance was similar for fruits between plants and fruits from the same plant (Table 3.2). The data indicate that the mature green fruits were not harvested at the same developmental stage, probably because defining the mature green stage visually was more difficult than for the ripe fruit and size alone is not a reliable indicator. Harvesting mature green fruit at different developmental stages could have a major effect on the firmness values, particularly since green fruit are firmer by at least an order of magnitude. A more reliable method for

green fruit would be to determine their age post-anthesis but this was not a practical option on such a large population.

Improvements in precision could be made by growing the parents and F_1 generation in the same glasshouse. This would allow a proper comparison of firmness between the parental plants and progeny and also allow an estimation of heritability of firmness between ITA 80-52-1 and Tamella. To allow an investigation into how ripe fruit firmness is affected by early fruit development the plants selected for extremes in ripe fruit firmness should be gown for another year and all fruit tagged from anthesis to allow harvesting of mature green fruit at a consistent stage. If there is a correlation between mature green fruit firmness and ripe fruit firmness then pools of mature green fruit could be used for further molecular analysis.

The high variation in the firmness data for mature green fruits means that few conclusions can drawn about the influence of texture in early fruit development upon the subsequent texture changes in ripe fruit. The differences in early fruit development could play a major role in determining any texture differences between cultivars. For the remainder of this thesis, the molecular analysis of texture determination will focus on ripe fruit.

The penetrometer used here was shown to be a reliable method of determining fruit firmness as also concluded by (Hietaranta and Linna, 1999). The maximum force measurement was consistent with the sensory evaluations of the parental plants. What

has to be remembered is that the parental plants were grown under different environmental conditions to those of the F_1 generation. The F_1 generation which was grown under glass and forced into early flowering, would have experienced significantly different temperatures and light conditions to the parental plants which were grown in poly-tunnels and flowered later. When the parental plants were moved from poly-tunnels to a glasshouse, the firmness values taken the following year were higher for each parent (Table 3.4) but still reflected the relative differences in texture of the fruits and were consistent with the sensory evaluation.

Another factor that may have influenced firmness was fruit size, as differences in cell size and number or differences in cell wall thickness due to cell wall swelling could affect texture (Redgwell *et al.* 1997). This was examined by an analysis of variance on fruit firmness values with fruit size as a covariate. Differences in the firmness of fruits from different plants were shown to be significant and this was further confirmed by analysing the relationship between firmness and fruit size in the entire F_1 population and in the twenty firmest and twenty softest plants. The slight negative correlation between fruit firmness and size for all of the F_1 plants is still obvious for the firmest fruit and extremely weak for the softest fruit. Thus at the extremes of the F_1 population, fruit size had little effect on the fruit firmness. The twenty softest and twenty firmest plants were selected to make up the fruit pools on the basis that the firmness values were reliable and sufficiently different for genetic analysis.

The success of the bulked analysis technique in characterising the genetic basis of a quantitative trait will depend on the magnitude of the phenotypic effect obtained. The larger the differences in firmness between the two populations the more marked the genetic differences related to fruit texture should be. In the case of the two pools here, the firmness of the least firm fruit in the firm pool is more than double the firmness of the firmest fruit in the soft pool. The choice of pool size will depend on the population size but is essentially empirical as the degree of background genetic variation is unknown. It will be a compromise between maximising the phenotypic differences and minimising the genetic background differences. The shape of the normal distribution (Figure 3.4) of fruit firmness suggests that texture is most likely to be a polygenic trait. Fruit texture exhibits a continuous variation (Figure 3.4) and bulked segregant analysis has been proposed as a way of analysing this type of complex genetic trait based on the screening of phenotypically informative individuals (Michelmore *et al.* 1991). When attempting to mark genes responsible for continuous variation (Wang and Paterson, 1994) suggested certain precautions should be taken. The first is to use crosses in which extreme variation is observed, which is the case in the cross of Tamella and ITA 80-52-1. Another is to use a large, and if possible, homozygous population. The analysis of 269 plants appears to be enough, but the use of a homozygous population is not possible for the cultivated strawberry. Not only is it octaploid, but it is also highly heterozygous. Attempts to obtain homozygous lines result in a high degree of inbreeding depression (Niemirowicz-Szczytt, 1989).

Quantitative variation, defined by a continuous phenotypic distribution in a segregating population, is caused by the combined effects of allelic variation at several loci (polygenic) and the environment. In this study the effect of the environment can be eliminated as a factor influencing firmness due to the experimental design and analysis. To detect and locate QTLs requires the generation of a segregating population, such as common backcross (BC) or F2 populations. The segregating population is characterised for molecular markers and associations between the genotypes and the molecular markers and phenotypes by means of specific statistical methods (Kearsey and Farquhar, 1998). Mapping QTLs is not as difficult as cloning the loci responsible for a QTL trait. There are currently efforts in cloning loci that affect photoperiodic induction of flowering, seed dormancy freezing tolerance and circadian period (Alonso-Blanco and Koornneef, 2000). The method generally used is chromosome walking, which requires the availability of complete physical maps of large genomic inserts (YACs and BACs) and a high precision map of the region of interest. This has been achieved with the QTL *fw2.2* which controls fruit weight between wild and cultivated tomato (Frary *et al.* 2000). The QTL *fw2.2* was located to a narrow chromosomal region ($1/10{,}000^{th}$ of the genome). The wild version of *fw2.2* was transformed into a cultivated tomato and the transformed fruit exhibited the expected weight decrease. This method is not suitable for octaploid strawberry as it is not possible to produce a mapping population with the cultivated strawberry and currently BAC or YAC libraries no not exist. It is hoped that combining bulked segregant analysis and cDNA subtraction will help in isolating genes which determine texture.

Chapter 4: ISOLATION OF TEXTURE RELATED GENES BY SUPPRESSIVE SUBTRACTIVE HYBRIDISATION.

4.1 INTRODUCTION

The identification of differentially expressed genes has helped in the understanding of gene function and the molecular events of biological processes. Differential screening (DS), subtractive hybridisation (SH) and differential display (DD) are the most commonly used methods for identifying differentially expressed genes.

Subtractive hybridisation is a method that produces small cDNA libraries enriched with transcripts that are present in only one of the compared samples. Using this for example, (Buchanan-Wollaston and Ainsworth, 1997) obtained a subtracted library in which 70% of clones were senescence-related. Subtractive hybridisation has also been used to compare genes expressed in red and green strawberry fruit (Medina-Escobar *et al.* 1997b) including a putative pectate lyase cDNA clone (Medina-Escobar *et al.* 1997a). The SH technique does have the disadvantage of isolating the more abundant transcripts and the detection of rare transcripts by this method requires the time-consuming analysis of many clones.

Differential display is a potentially powerful technique to identify differentially expressed genes. This method uses PCR to amplify a subset of cDNA in a population using a specific set of short oligonucleotide primers. The resultant cDNA fragments are separated and displayed by denaturing polyacrylaminde gel electrophoresis. Wilkinson *et al.* (1996) used DD to isolate five cDNA clones that were up regulated in ripening strawberry fruit. Three of these cDNAs were fruit specific. Although this technique has

undergone a number of improvements (Wan *et al.* 1996) there have been few reports of large numbers of clones being isolated by this method.

A range of different enzymes have been shown to act on cell walls (Fry, 1995). Different fruits do not share the same softening mechanism. In tomato high levels of polygalacturonase accumulate during softening (Brady, 1987) and endoglucanase is low (Maclachlan and Brady, 1992). In strawberry PG activity is absent (Huber, 1984) while endoglucanase is easily detectable (Abeles and Takeda, 1990). The molecular basis of softening in ripening strawberry fruit is poorly understood and only a few genes with cell wall associated functions have been characterised. No cell wall degrading enzymes have been convincingly linked to fruit firmness. Increased cellulase activity has been correlated with firmness (Abeles and Takeda, 1990) and cellulase is up-regulated at the turning stage in ripening strawberry fruit (Manning, 1998). Other possible firmness related genes are pectate lyase and PME whose activity increases in ripening strawberry fruits (Barnes and Pratchett, 1976).

In an attempt to focus on genes that might be more specifically related to texture, molecular studies were designed to exploit the F_1 strawberry population segregating in fruit firmness. Pools of firm and soft fruits were produced and used for comparing gene expression. In this way pooling was expected to minimise the differences in genetic background which, are not associated with texture. This strategy aims to create pools in which differences in gene expression only relate to firmness. To clone these genes a novel technique called suppressive subtractive hybridisation (SSH) (Diatchenko *et al.* 1996) was used. This method has been used to identify tissue-specific genes from the human Y chromosome (Diatchenko *et al.* 1996) and genes involved in T-cell activation (Gurskaya *et al.* 1996). Other uses of this technique include investigations of the differences between developing queen and worker honeybees (Evans and Wheeler, 1999); endoderm formation in *Xenopus* (Hudson *et al.* 1997) and tumour necrosis factor-induced cell death (Chu *et al.* 1997).

4.2 METHODS

Total RNA was isolated from each of the pools (ripe fruits from 5, 10 and 20 individual plants) and translated *in vitro* using ^{35}S-methionine to label the translated polypeptides. The polypeptides were separated by 2-D-polyacrylamide gel electrophoresis (2D-PAGE) and visualised by fluorography.

Poly $(A)^+$ mRNA was isolated from the total RNA from the pools of ripe fruit from twenty individual plants and used for cDNA preparation. The subtraction, cloning, screening and sequencing was carried out as described in chapter 2.

4.3 RESULTS

4.3.1 2-D-PAGE analysis of *in vitro* translation products from firm and soft fruit pools

As a first step to examining the differences in gene expression between the pools, *in vitro* translation products were analysed by 2-D PAGE. The ^{35}S-methionine labelled polypeptide translation products were separated by 2-D gel electrophoresis. The 2-D-gel electrophoresis could have given a good indication of how many differences there were between the soft and firm pools and whether there would be any visible difference between the five, ten and twenty pools. Unfortunately, the resolutions of the gels were very poor, making it impossible to compare the different pools (Fig. 4.1).

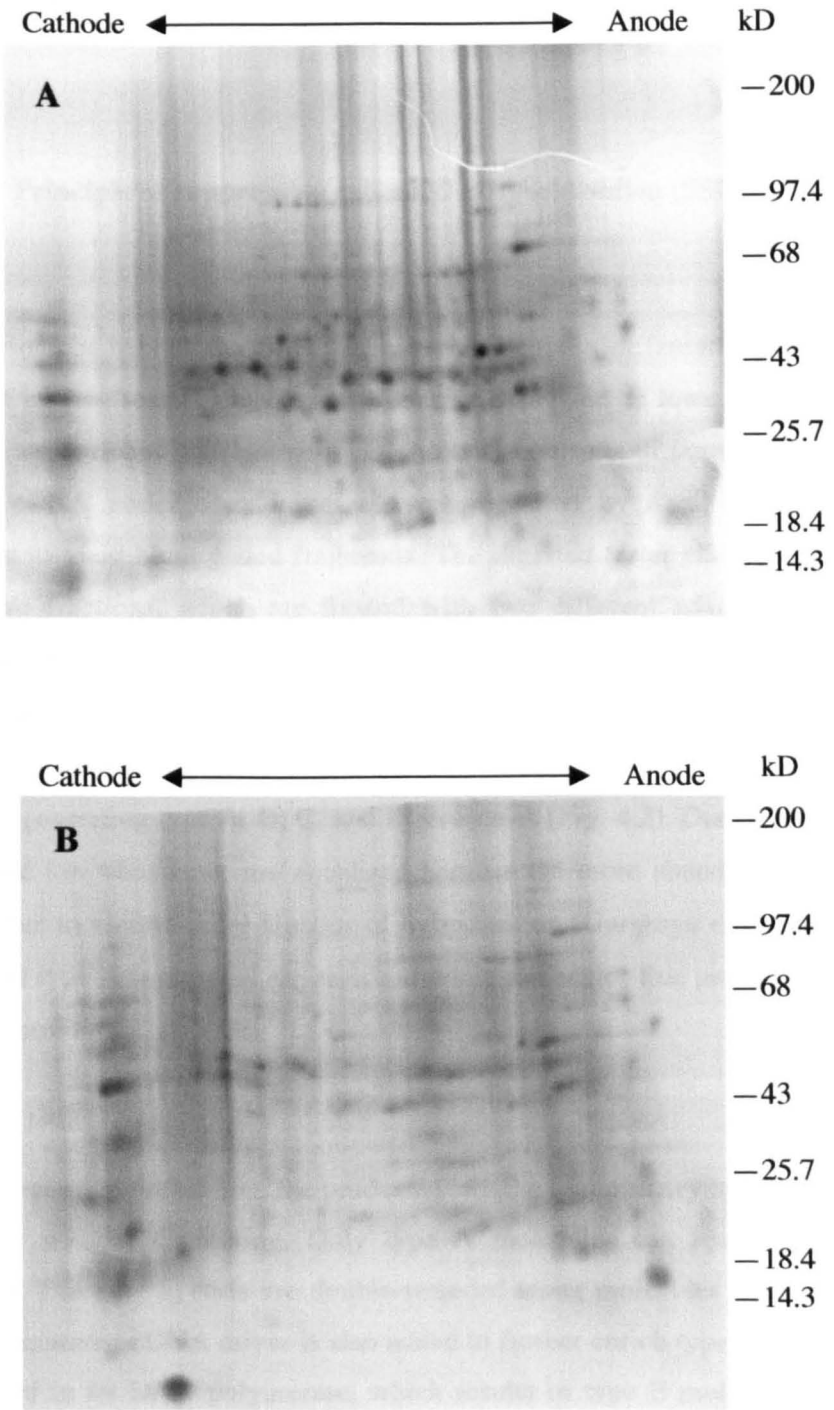

Figure 4.1 Comparison of *in vitro* translation products from firm pool (5 plants) (A) and soft pool (5 plants) (B). Total RNA was isolated from both pools and translated *in vitro* in a rabbit reticulocyte system. ^{35}S-labelled polypetides were separated by two-dimensional polyacrylamide gel-electrophoresis using isoelectric focusing in the first dimension (left to right) and size separation in the presence of SDS in the second dimension (top to bottom).

4.3.2 Principle of suppression subtractive hybridisation (SSH)

The principle behind the SSH technique is that the differentially expressed cDNAs present in the "tester" cDNA but absent or expressed at lower levels in the "driver" cDNA are enriched by removing cDNAs common to both populations. The tester and driver cDNA undergo an initial enzyme digestion by *RsaI*, a four-base restriction enzyme to yield blunt ended fragments. The digested tester cDNA is then sub-divided into two fractions, which are ligated with two different adapters. The ends of the adapters are unphosphorylated so only one strand of each adapter covalently attaches to the 5' ends of the tester cDNA. In the first of two hybridsations, an excess of driver is added to each tester cDNA fraction. The two mixtures are heat denatured and allowed to anneal, generating type A, B, C, and D molecules (Fig. 4.2). During this step cDNAs of high and low abundance are equalised because the more abundant molecules reanneal faster due to second-order kinetics of hybridisation (Gurskaya *et al.* 1996). The single-stranded type A molecules are enriched while molecules that are common to tester and driver form type C hybrids.

In the second hybridisation, the products from the two primary hybridisations are mixed together without denaturing. Only type A molecules can reanneal to from type E hybrids. The new hybrids are double-stranded tester molecules with asymmetric ends. Fresh denatured cDNA driver is also added to further enrich type E molecules. The ends are filled in by DNA polymerase, which results in type E molecules having different annealing sites on their 5' and 3' ends. The hybridisation mixture is subjected to PCR with primers P1 and P2 (Table 2.2) that correspond to the outer parts of the adapters 1 and 2, respectively. Exponential amplification can only occur with molecules of type E. Molecule C is amplified linearly and molecules B and D are not amplified. Molecule B is not amplified as it has long inverted repeats on the ends that form a stable "panhandle

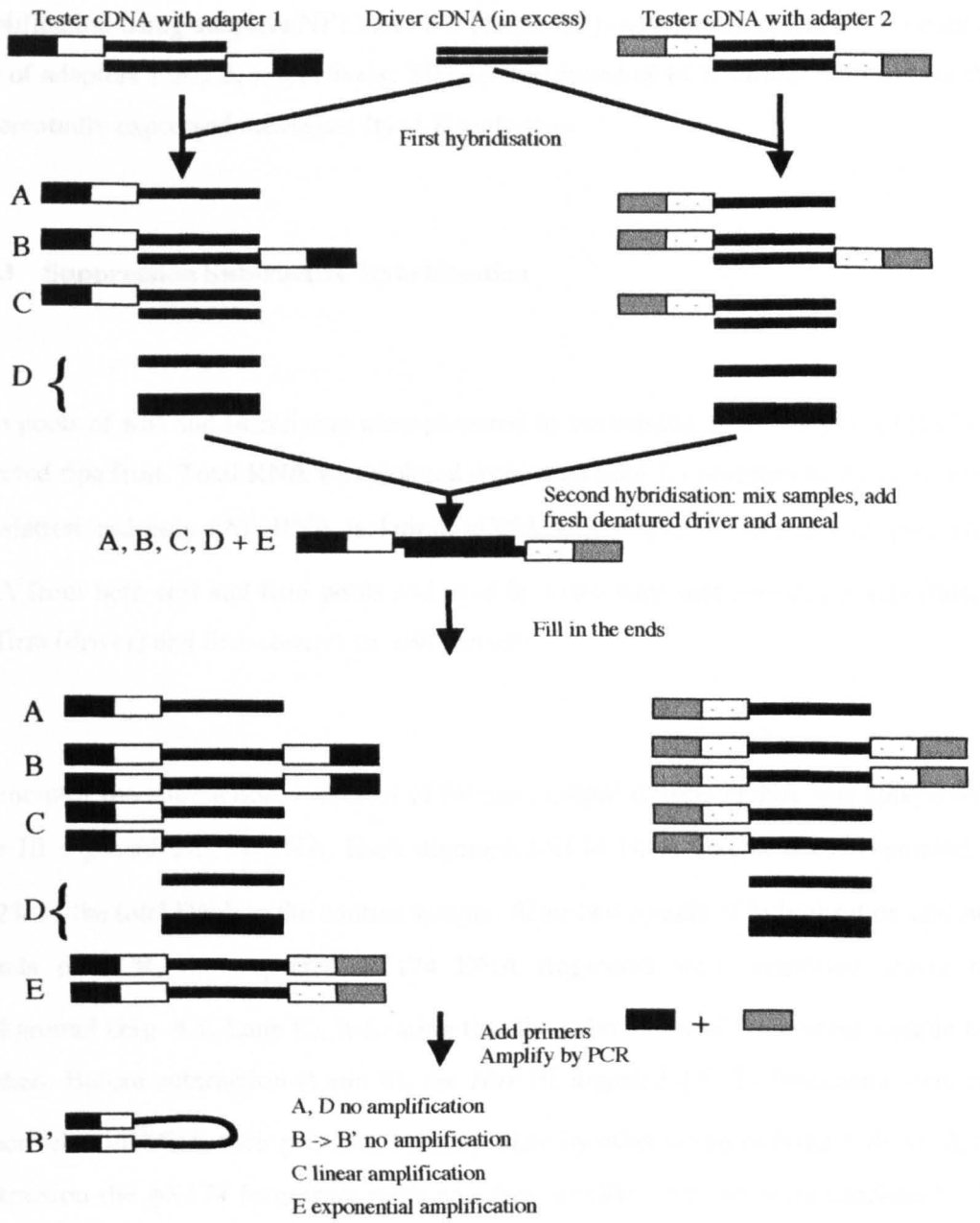

Figure 4.2 Scheme of suppression subtractive hybridisation (Diachenko *et al*, 1996). Sequences would be isolated from the firm pool by using firm pool cDNA as tester and soft pool cDNA as driver. Sequences would be isolated from the soft pool by using soft pool cDNA as tester and firm pool cDNA as driver.

-like" structure (Siebert *et al.* 1995). The mixture is subjected to a second round of amplification using adapters NP1 and NP2 (Table 2.2), which correspond with the inner part of adapters 1 and 2, respectively. This second round of PCR further enriches for the differentially expressed messages, (type E molecule).

4.3.3 Suppression Subtractive Hybridisation

Two pools of soft and firm tissue were prepared by combining equal weights (3g) of the selected ripe fruit. Total RNA was isolated from each pool for northern analysis, *in-vitro* translation and poly $(A)^+$ RNA isolation. cDNA was prepared from 2µg of poly $(A)^+$ RNA from both soft and firm pools and used in a two-way subtraction, i.e. soft (tester) vs. firm (driver) and firm (tester) vs. soft (driver).

To monitor the subtraction, a control of human skeletal muscle cDNA was spiked with *Hae* III digested ϕX174 DNA. Each digested ϕX174 DNA fragment corresponded to 0.02% of the total DNA in the control sample. After two rounds of hybridisation and two rounds of PCR, the expected ϕX174 DNA fragments were amplified above the background (Fig. 4.3, Lane E), indicating that the subtraction of the control sample had worked. Before subtraction (Lane F), the *Hae* III digested ϕX174 fragments were not selectively amplified, their presence being masked by other co-amplifying cDNAs. After subtraction the ϕX174 fragments were enriched significantly and were amplified. The majority of the human skeletal muscle cDNA had been removed by two rounds of subtraction. The bands were identical to the supplied control, (Lane G) albeit slightly larger due to the ligated adapters.

The results for the soft and firm subtractions were not so clear. After the primary and secondary PCR reactions, there were no visible differences between the subtracted and unsubtracted samples nor any discretely amplified products for both the firm and soft cDNA samples. The unsubtracted cDNA, which is amplified because it contains molecules with adapters 1 and 2, appears as a smear between 1.5kB and 100bp. A subtracted cDNA molecule also can only be amplified if it contains adapters 1 and 2, i.e. it formed a single stranded molecule with adapter 1 after the first hybridisation and after the second hybridisation it annealed with a complementary single stranded tester molecule with adapter 2. Theoretically these are the differentially expressed sequences. The continuous smears from 1.5kb to 100bp (Fig. 4.3) indicate either that there are many different cDNAs between the pooled soft and firm populations or that non-specific amplification has occurred.

In order to identify amplified products the subtracted cDNAs from both pools were cloned into the pPCR-Script vector and white colonies were analysed by PCR using M13 forward and reverse primers to determine the size of the inserts. Eighty clones were randomly selected from each subtracted pool for differential screening. The PCR products of the soft and firm clones were dot-blotted onto duplicate filters. To detect cDNAs expressed at low levels, the clones were hybridised with both subtracted soft and firm cDNA. Before the subtracted cDNA was used as a probe, the adapters were digested away with *RsaI*, to minimise background hybridisation. The adapters were removed by using spin columns and the cDNA probe was precipitated by ethanol.

Almost all of the firm clones examined contained DNA that hybridised with the subtracted firm cDNA probe and similarly almost all of the firm specific clones hybridised with the subtracted soft cDNA probe (Fig. 4.4). The overall signal with the subtracted firm cDNA probe was higher than the subtracted soft cDNA probe, which made it difficult to identify clones with genuine differences in expression. Serial dilutions of both probes were dot-blotted onto each membrane as a control and to aid in

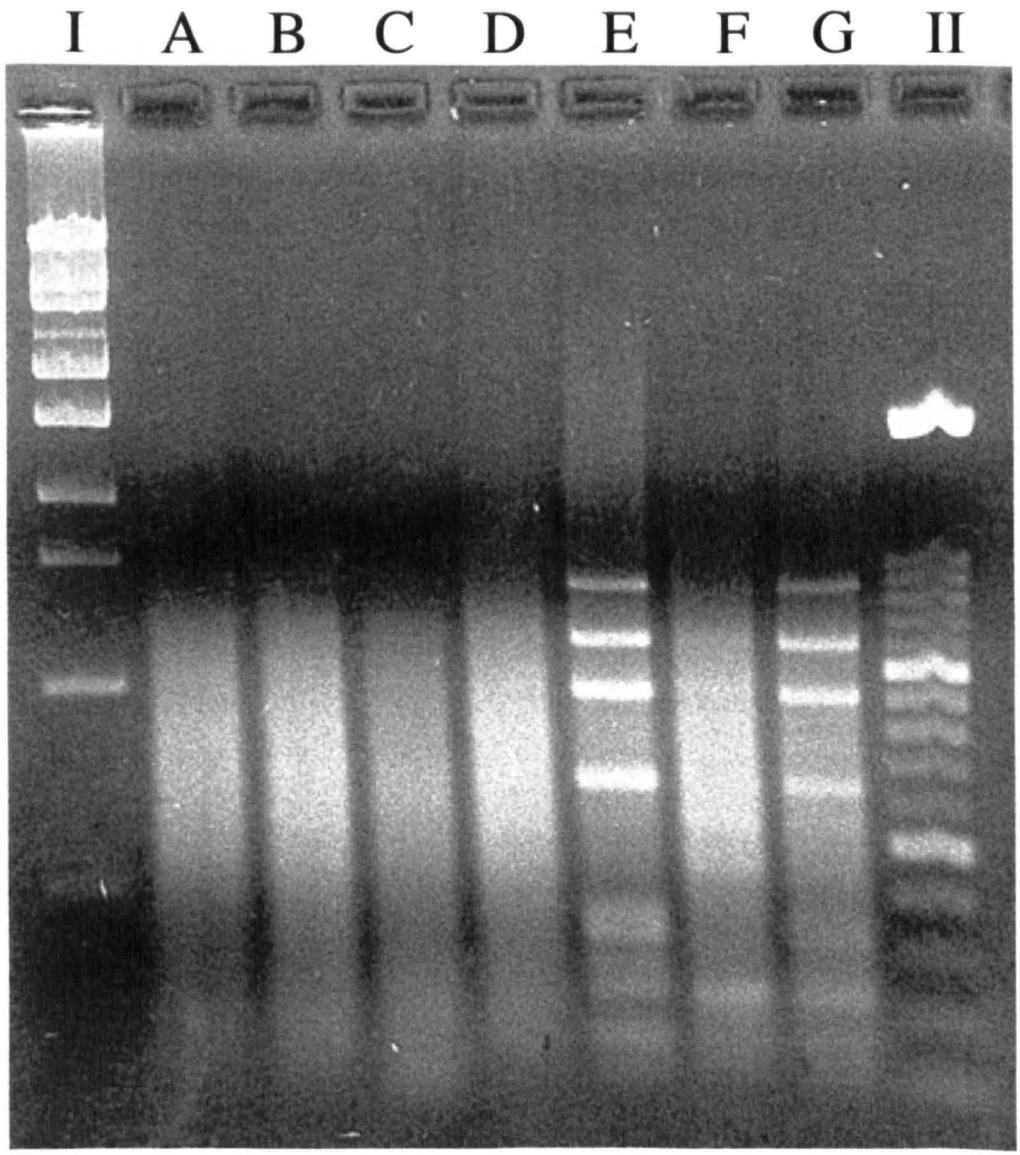

Fig.ure 4.3 Analysis of suppressive subtractive hybridisation. Lane A - subtracted firm cDNA; Lane B-unsubtraced firm cDNA; Lane C-subtracted soft cDNA; Lane D - unsubtracted soft cDNA; Lane E - subtracted skeletal muscle cDNA; Lane F - unsubtracted skeletal muscle cDNA; Lane G - kit supplied subtracted skeletal muscle cDNA. Amplified cDNAs were separated on a 2% (w/v) agarose gel with size makers in lane I - StyI/Hind III and lane II - 100bp ladder.

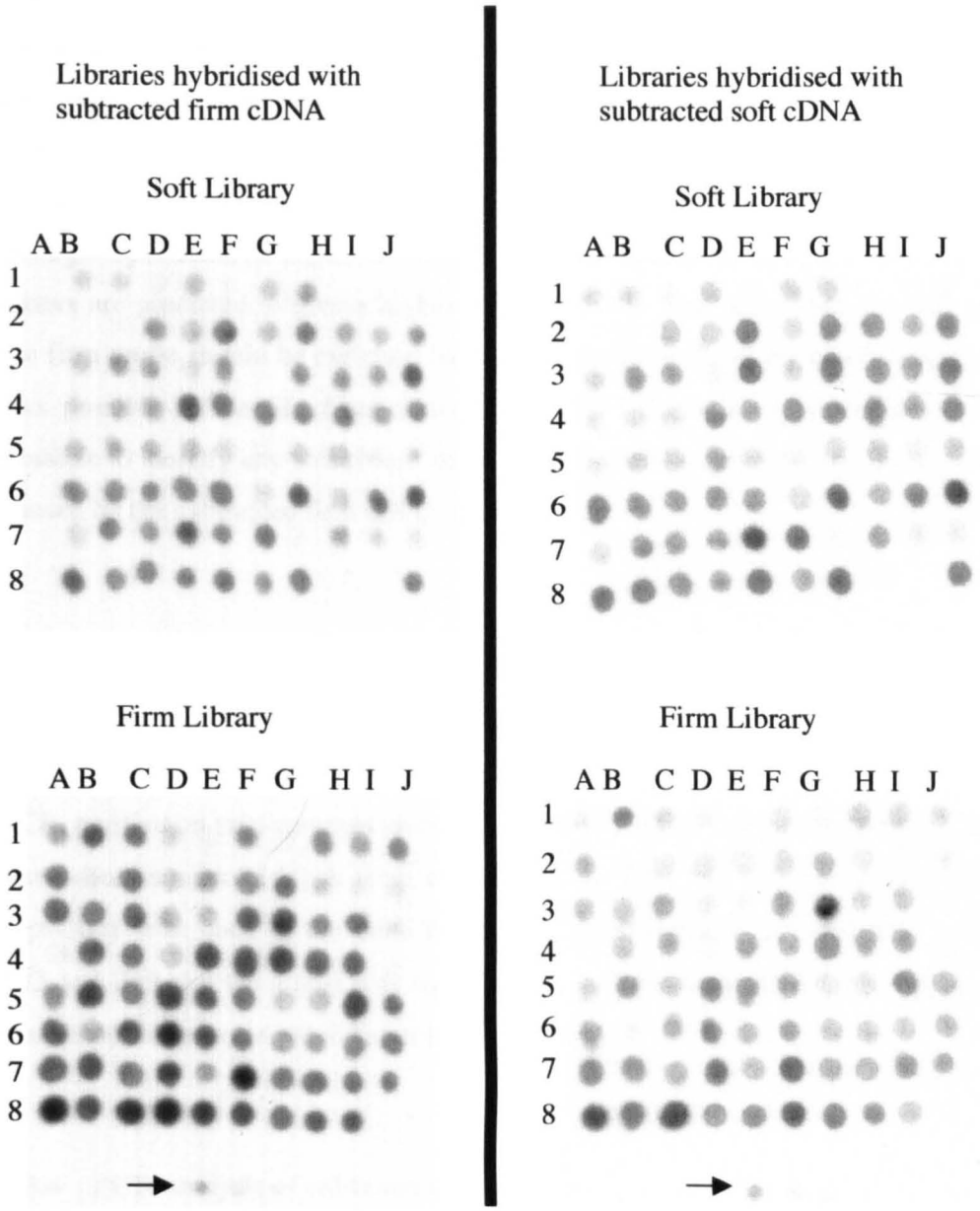

Figure 4.4 Differential screen of selected PCR products sub-cloned from subtracted soft and firm pools. One micro-litre of denatured PCR product was applied to duplicate nylon membranes. The membranes were hybridised with ^{32}P-labelled *RsaI* digested subtracted soft and firm cDNA. Arrows indicate hybridisation signals of serial dilution of *RsaI* digested subtracted firm cDNA.

determining the correct exposure time. Surprisingly, no hybridisation was obtained with the serially diluted dot blots of soft subtracted DNA when probed with either itself or the firm probe, suggesting that the dot-blotted cDNA was not fixed to the filter. Both the soft and firm probes hybridised to the serially diluted samples of the firm probe. After adjusting the exposure of the two filters, it was evident that the firm probe produced a stronger hybridisation signal to the soft and firm clones than the soft probe. The firm clones are predicted to give a higher signal with the firm probe than the soft probe, as the firm probe should be enriched for clones specific to firm. By careful comparison, it was possible to identify firm clones that show differential hybridisation. It was not possible to identify any soft clones showing differential expression due the higher signal, caused by the subtracted firm cDNA probe.

Clones from the subtracted firm library in positions J1 (clone F13), C2 (clone F17), E2 (clone F19), D4 (clone F52) and A8 (clone F95) (Figure 4.4) were chosen for further characterisation by northern analysis. Inserts from the selected clones were amplified by PCR, purified on spin columns and labelled with ^{32}P-dCTP by random priming. Northern blots containing total RNA from ripe fruit from firm and soft pools were hybridised separately with each of the three clones. Hybridisation was detected only with clones F13 and F19 and the signal was equal for the soft and firm RNA (Fig. 4.5), indicating that these clones were not differentially expressed.

4.3.4 PCR analysis of subtraction efficiency

The fact that only two of the five selected clones produced detectable hybridisation to RNA and these did not show differential expression, suggests the subtraction had failed or at least was inefficient for the firm and soft cDNAs. One method to test the efficiency of subtraction is to compare the abundance of a cDNA with similar expression in firm and soft fruit before and after subtraction. Ripening-related strawberry genes not

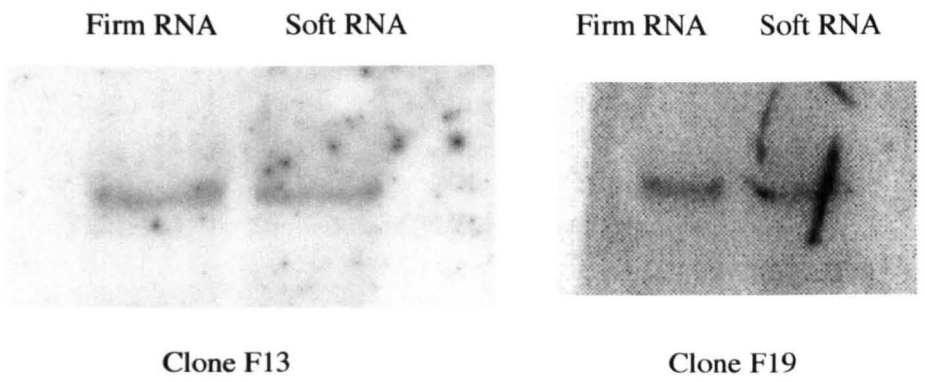

Figure 4.5 Northern analysis of strawberry RNA with clones F13 and F19 as probes. Total RNA (10μg per lane) was fractionated by denaturing agarose gel electrophoresis, transferred to nylon membranes, and hybridised with radiolabelled cDNA probes.

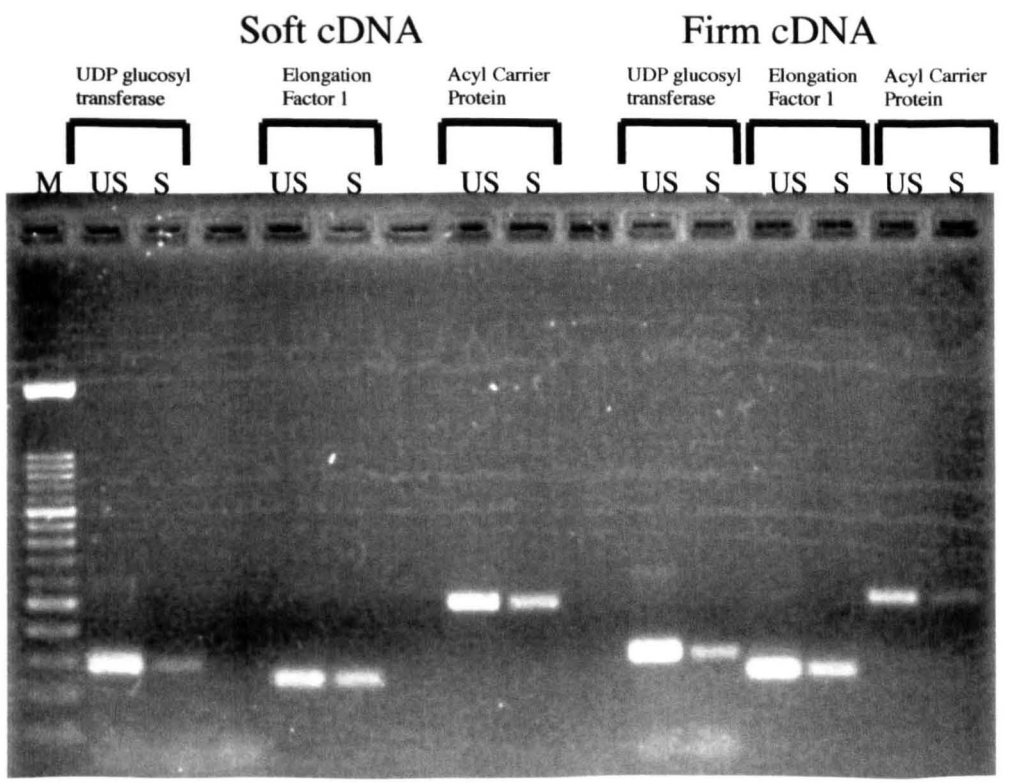

Figure 4.6 PCR amplification of selected cDNAs from unsubtracted (US) and subtracted (S) firm and soft cDNA. PCR was performed using gene specific primers and only for 20 cycles. The PCR products were run on a 2% agarose gel with a 100bp marker (M).

Table 4.1 Sequences of PCR primers for selected clones

Primer	Sequence
	UDP_glucosyl transferase
74/3	CACGTATGCTTCACCAGATGGG
74/ R1	CGGCGATGGTCTTGTTACTGATCC
	Elongation Factor
EF33A4	GTTGAGGATGTTCCCTGTGGTAAC
EF33AR3	GACAACCATAGGAATCGGACTTAGC
	Acyl Carrier Protein
ACP3	CTCAAGCCACTATCAGGTTC
ACP4	GGGTCAATGGTCTTAAGCCA

associated with cell wall functions would be predicted to have similar expression in the firm and soft pools and these could be used to test subtraction efficiency. The previously identified strawberry cDNAs encoding UDP glucosyl transferase, elongation factor I and acyl carrier protein (Manning, 1998), were estimated semi-quantitatively in the unsubtracted and subtracted soft and firm samples by PCR using the primers described in Table 4.1 and 20 cycles of amplification. The results in Figure 4.6 shows that PCR products of the expected sizes were obtained from both soft and firm pooled samples and that considerably less of the corresponding cDNA was present after subtraction. This indicates that the subtraction is partially successful but not as complete as expected with the consequence that many of the cloned cDNA do not represent differentially expressed genes.

4.3.5 Differential screening using unsubtracted cDNA probes

A second approach was used to identify differentially expressed genes in pools of firm and soft cDNAs. Unsubtracted cDNA was used directly as a probe. The cDNAs cloned from the original subtractions were dot-blotted onto duplicate membranes for the second time. The membranes were hybridised with ^{32}P-labelled firm and soft-pooled ds cDNA. The soft cDNA probe hybridised with the soft and firm clones more strongly than the firm cDNA probe (Fig. 4.7). The soft probe hybridised more strongly to the pooled firm cDNA than the firm probe (Fig 4.7). The pattern of hybridisation was different to that of the first screen with subtracted probes. Hybridisation signals with these probes exhibited much greater variation between clones than that observed with the subtracted probes (Fig 4.4) and less than half of the firm and soft clones produced a detectable signal. Two reasons can be proposed to explain these differences. Firstly, the probes used in the second screen had not been normalised and hence are less sensitive for detecting the rarer mRNAs. Secondly, if the removal of adapters from the probes used in the first screen was incomplete there could have been cross-hybridisation between the adapter sequences. Twenty putative differentially expressed clones were selected for sequencing

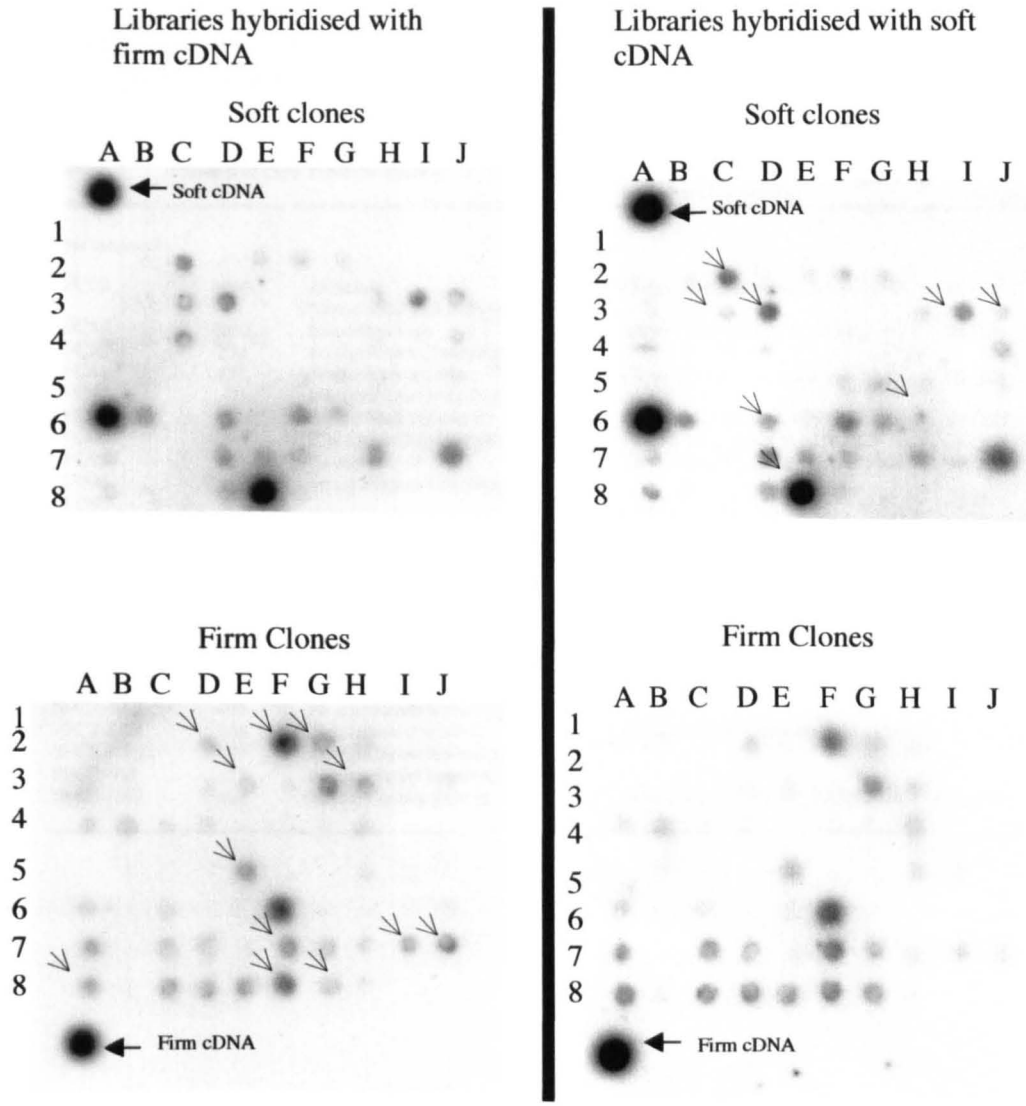

Figure 4.7 Differential screen of cloned PCR products from subtracted soft and firm pools. One micro-litre of denatured PCR product was applied to duplicate nylon membranes. One set of firm and soft clones was hybridised with ^{32}P-labelled *RsaI* digested soft cDNA and another set of firm and soft clones was hybridised with ^{32}P-labelled *RsaI* digested firm cDNA. Thick arrows indicate *RsaI* digested cDNA. Thin arrows indicate clones selcted for sequencing.

Table 4.2 Characterization of selected cDNA clones from firm and soft subtractions. Homology is based on amino acid identities with length of compared translated sequence in brackets. See appendices IV and V for sequences and alignments.

Clone	Clone size (bp)	Putative identity	Related sequence and accession number	% homology (length)
Firm enhanced				
FFC18	1060	Invertase	*Fragaria x ananassa* AAD10960	66 (136)
FFC20	1052	Glyceraldehyde-3-phosphate dehydrogenase	*Antirrhinum majus* P25861	94 (79)
FFC22	274	no significant homology		
FFC41	177	Heat Shock protein	*Picea mariana* AAC32131	98 (50)
FFC47		no significant homology		
FFC65	1068	Heat Shock protein 80	*Euphorbia esula* AAF31705	63 (187)
FFC89	253	Flavanone 3-hydroxylase	*Persea americana* AAC97525	78 (38)
FFC95	413	Aconitase	*Arabidopsis thaliana* CAA21469	90 (132)
FFC96	358	no significant homology		
FFC97	no sequence			
FFC104	454	Malic Enzyme	*Solanum tuberosum* P37225	67 (103)
FFC105	988	Ubiquitin-conjugating enzyme	*Arabidopsis thaliana* AAA8664	72 (68)
Soft enhanced				
SFC3-3.S4	324	Cinnamyl-alcohol dehydrogenase	*Fragaria x ananassa* AAD10327	100 (22)
SFC3-3 1	448	60S Ribosomal protein L10	*Euphorbia esula* AAF34765	98 (60)
SFC3-3.52	195	60S Ribosomal protein L10	*Euphorbia esula* AAF34765	91 (36)
SFC2-48	466	no significant homology		
SFC3-3.88	155	26S Ribosomal RNA	*Lycopersicum esculentum* X13557	99 (blastn)
SFC3-3.S12	557	no significant homology		
SFC3-S15	177	no significant homology		
SFC3-3.82	468	WD-40 repeat protein	*Arabidopsis thaliana* BAB02018	79 (77)

and northern analysis on the basis of apparent hybridisation differences between the firm and soft probes. The results of the homology search are summarised in Table 4.2.

4.3.5.1 Clones for mRNAs with apparent enhanced expression in firm fruit

The cDNA clone FFC18, which was isolated from the firm fruit pool, showed a strong homology to invertase. The predicted amino acid sequence from a 1kb region was ~66% identical to translated sequences from invertase cDNAs from strawberry (AAD10959 and AAD10960). The clone shows a high similarity to the 3' ends of both the invertase homologues (Figure 4.10). The expression of the cDNA clone FFC18 was similar in the firm and soft pools (Fig. 4.9). This clone showed a higher signal with the soft pool cDNA in the differential screen although the firm pool probe gave a higher overall signal to most clones.

Three clones showed homology to enzymes of the tricarboxylic acid cycle and glycolysis pathway. The cDNA clone FFC20 showed a 94% amino acid identity along a 1kb region to a cDNA GPDH clone (P25861) from *Antirrhinum majus*. FFC20 was also similar to clones from *Dianthus caryophyllus* (P34921) and *Atriplex nummularia* (CAA53269). Northern analysis with this clone as a probe showed that it had higher transcript levels in the soft pool (Fig. 4.9). This was also the case with the differential screen where the clones showed a higher signal with the soft pool cDNA probe. The cDNA clone FFC104 showed a 67% amino acid identity to a malic enzyme homologue from *Solanum tuberosum* (P37225). Other very similar clones were AAC13636 and CAB80866 both from *Arabidopsis thaliana*. No hybridisation signal could be detected by northern analysis. This was surprising because the clone was detected in the differential screen although the signal with the soft cDNA probe was very weak. A signal was detected on a "virtual" northern (based on PCR) after a 12-day exposure using the FFC104 clone as a probe. A higher signal was obtained with the soft cDNA (Fig. 4.9). This contradicted the results from differential screen, which showed a higher

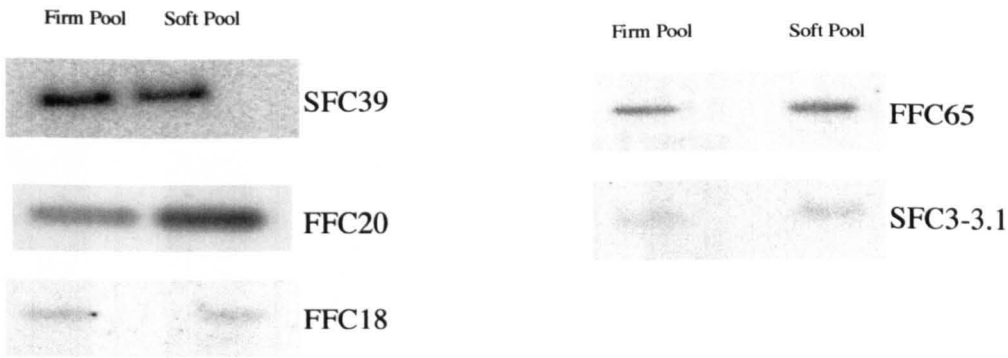

Figure 4.8 Northern-blot analysis of strawberry RNA probed with selected clones from the second differential screen. Total RNA (10μg per lane) was fractionated by denaturing agarose gel electrophoresis, blotted to nylon membranes, and hybridised with radiolabelled cDNA probes (indicated on the right of each panel).

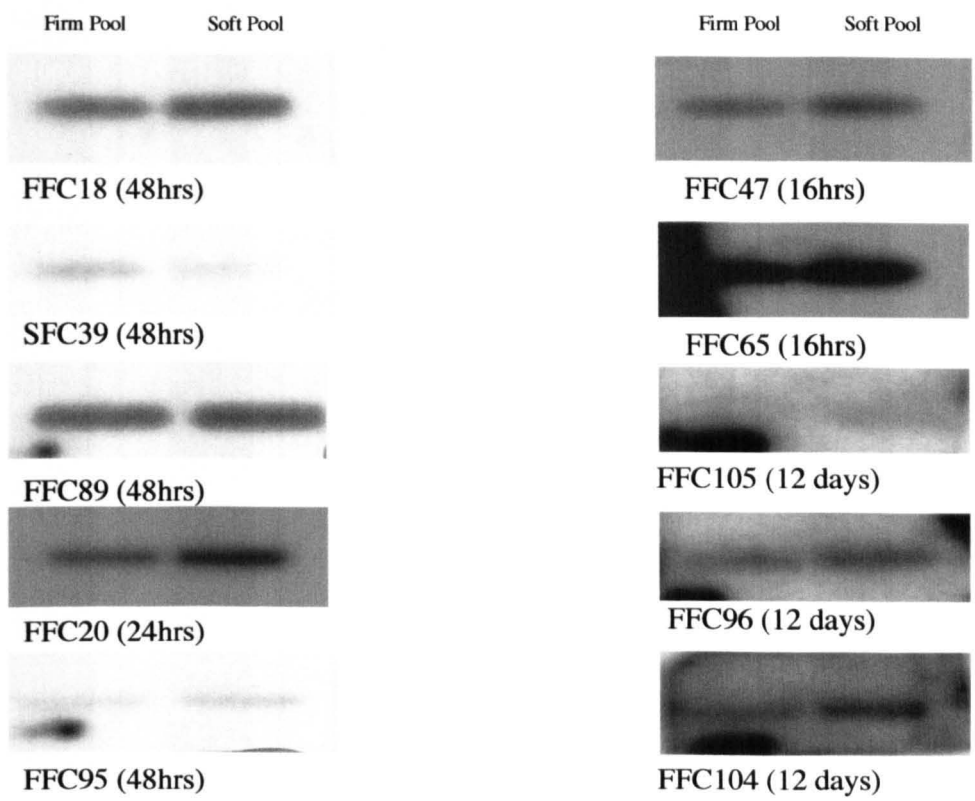

Figure 4.9 Virtual northern analysis of selected clones from the second differential screen. cDNA from both pools used for AFLP was amplified by *Mse*I-core and *Eco*RI-core primers for 25 cycles. The PCR products were partially fractionated by agarose gel electrophoresis, blotted to nylon membranes, and hybridised with radiolabeled cDNA probes indicated below each panel.

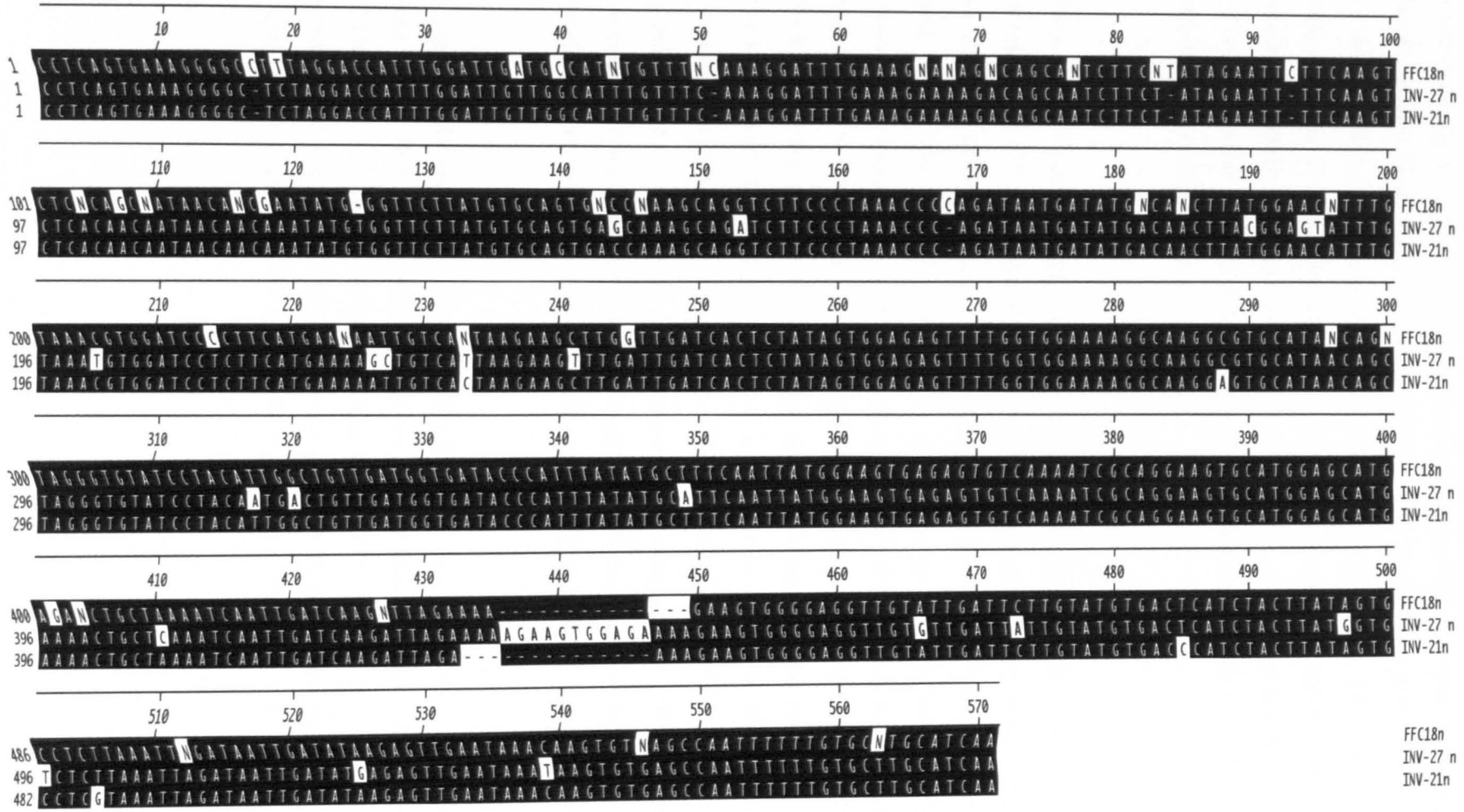

Figure 4.10 Alignment of the putative invertase clone, FFC18, with the invertase clones INV-21 (AD10959) and INV-27 (AD10960) from GenBank.

signal with the firm cDNA. The results indicate that the FFC104 clone has low transcript levels in ripe fruit. Another cDNA, FFC95, had 90% amino acid identity with an aconitase cDNA clone from *Arabidopsis thaliana* (CAA21469). The clone also shows high similarity to clones isolated from *Solanum tuberosum* (CAA65735) and *Cucurbita maxima* (P49608). Aconitase catalyses the conversion of citrate to isocitrate in the TCA cycle. The virtual northern (Fig. 4.9) showed a higher signal with the soft cDNA probe although the overall signal was weak.

Two clones isolated from the firm cDNA subtraction encode putative heat shock proteins. The cDNA clone FFC41 had a 98% amino acid identity with a putative Heat Shock 80 (HS80) clone from *Picea mariana* (AAC32131). The HS80 is a member of a gene family that is highly conserved in a wide range of plant species. The second putative heat shock protein was encoded by clone FFC65, which showed 63% amino acid identity to a putative heat shock protein from *Euphorbia esula* (AAF31705). The FFC65 clone has a higher transcript level in the soft pool than the firm pool (Fig. 4.8). The FFC41 and FFC65 clones appear to be similar as both show high homology to a clone isolated from *Lycopersicon esculentum* (P36181) that shows increasing expression in developing ovaries, floral shoots and roots (Koning *et al.* 1992) but the levels decrease with maturation. The FFC41 and FFC65 sequences weakly align, but this comparison is hampered by the short and poor quality of the sequence obtained.

A putative flavanone-3-hydroxylase (F3H) cDNA clone (FFC89) was isolated. The clone had a 78% amino acid identity to a clone from avocado (*Persea* americana, AAC97525). A database search did not identify any F3H clones previously isolated from strawberry by Manning (1998). A virtual northern (Fig. 4.9) of the clone showed a slightly higher signal with the soft cDNA than the firm cDNA probes.

The cDNA clone FFC105 had 72% amino acid identity to an ubiquitin-conjugating enzyme from *Arabipodsis thaliana* (AAF8664). The Ubiquitin-conjugating enzyme is part of the ubiquitinylation process. The transcript level of FFC105 in the firm and soft pool was investigated by virtual northerns. After an exposure of 48 hours, no signal could be detected, but a weak signal could be detected after an exposure of 12 days (Fig. 4.9). This indicates that there is a very low level of transcript in the two pools. The differential screen (Fig 4.7) showed a difference between the signal obtained from this clone from the firm and soft probes, but it is difficult to interpret the results, due to the non-equal signal activity of the two probes.

Two other cDNA clones, FFC96 and FFC47 did not have any appreciable homology to the database sequences. The cDNA clone FFC47 showed higher transcript levels in the soft pool than the firm pool (Fig. 4.9). After an exposure of 12 days, the gene encoding FFC96 was observed to be expressed at much lower levels in firm fruit (Fig. 4.9).

4.3.5.2 Clones for mRNAs with apparent enhanced expression in soft fruit

The first clone from the putative soft enhanced group to be analysed was SFC3-3.S4. This clone had 100% amino acid identity with a cinnamyl-alcohol dehydrogenase (CAD) homologue (AAD10327) isolated from *Fragaria x ananassa*. The perfect homology indicates that the cDNA clone SFC3-3.S4 and the CAD are identical. Northern analysis showed a marginally higher transcript level in the firm pool than the soft pool (Fig. 4.8). Similar results were obtained with a virtual northern (Fig. 4.9). CAD converts the cinnamyl aldehydes into cinnamyl alcohols which are the monomeric precursors of lignin (Higuchi, 1985).

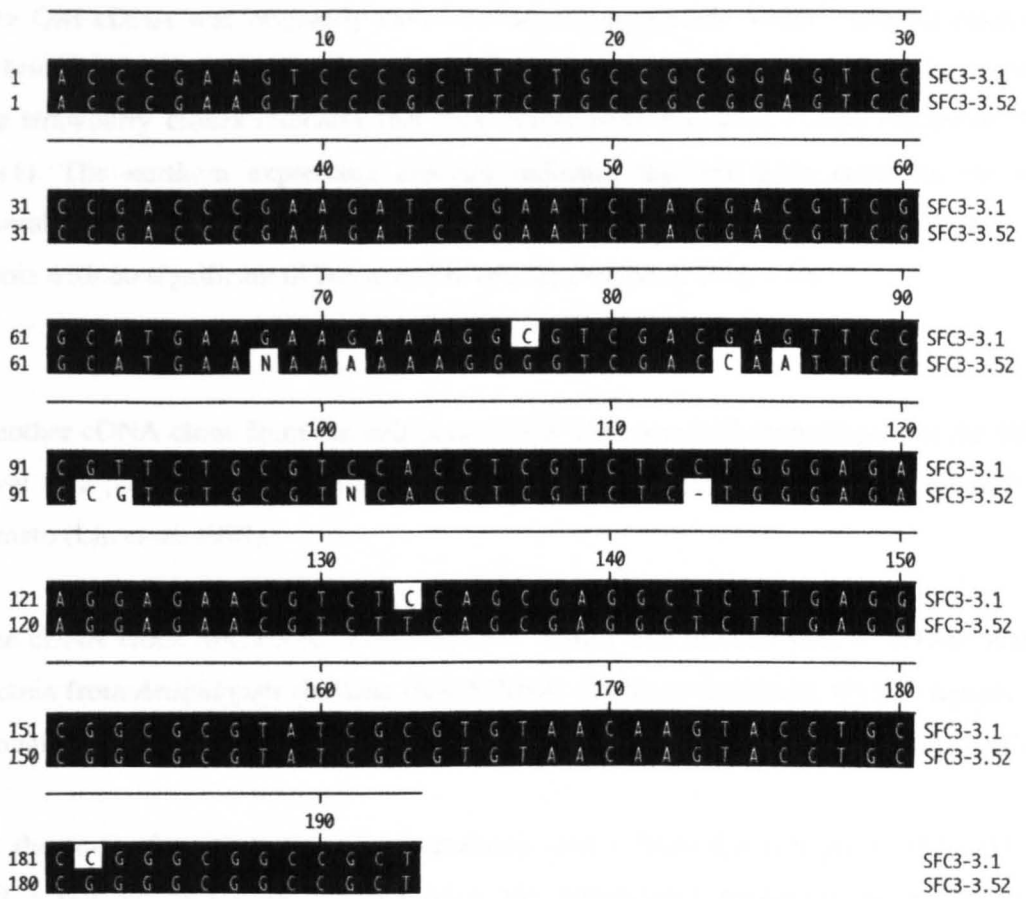

Figure 4.11 Alignment of the putative QM clones SFC3-3.1 and SFC3-3.52. QM is a novel gene that was originally identified as a putative tumour suppresser gene. N is an unidentified base.

Two cDNA clones, SFC3-3.1 and SFC3-3.52, exhibit 98% amino acid identity with a QM, a putative transcription factor, homologue from *Euphorbia esula* (AAF34765) isolated from underground adventitious buds. Genes encoding the QM family are highly conserved and have been found in organisms from yeast to humans (Nika *et al.* 1997). The QM cDNA was originally isolated from a non-tumeric Wilm's tumour microcell hybrid and is thought to be a tumour suppresser (Koller *et al.* 1996). A comparison of the strawberry clones indicates that they derive from the same cDNA transcript (Fig. 4.11). The northern expression analysis indicates that the gene encoding the QM homologue (SFC3-3.1) is expressed at low levels in ripe fruit from the firm and soft pools with no significant difference between the two pools (Fig. 4.8).

Another cDNA clone from the soft pool, SFC3-S88, was 99% homologous at the DNA level to a putative 26S ribosomal RNA from *Humulus lupulus* and a 25S clone from tomato (Lin *et al.* 1999).

The cDNA clone SFC3-3.82, exhibited 79% amino acid identity with A WD-40 repeat protein from *Arabidopsis thaliana* (BAB02018). Proteins containing WD-40 repeats are thought to be involved in protein-protein interactions (Vandervoorn and Ploegh, 1992).

At the time of writing, the three remaining clones from the soft pool (SFC3-3.S12, SFC3-S15 and SFC2-48) did not have any appreciable homology to the database sequences.

4.4 DISCUSSION

The clones from the subtraction were initially screened with firm subtracted probe and soft subtracted probe. The lack of success in determining which clones were differentially expressed could be due to the incomplete digestion and removal of the adapter sequences. The adapter sequences could have caused a very high background when the dot blots were hybridised with the firm and soft probes. The firm and soft probes obtained by subtraction should enable the identification of low-abundance differentially expressed sequences, as the probes should be enriched for these sequences. Probes derived from unsubtracted "driver" and "tester" cDNA may not detect the low abundance sequences. The results of the dot blots and northern analysis do not show that there has been any significant enrichment after subtraction. The level of enrichment will depend upon how efficiently non-differentially expressed sequences are subtracted. The removal of common sequences in the "driver" and "tester" populations depends on the relative concentrations between the two pools. The subtraction efficiency of the method employed can be assessed by comparing the abundance of known cDNAs before and after subtraction. Ideally, this is done with both non-differentially and differentially expressed genes between the two RNA sources being compared. The subtracted and unsubtracted products can also be examined by Southern hybridisation. Gurskaya *et al...* (1996) evaluated the subtraction efficiency of DNA populations from activated and non-activated T-cells by probing the subtracted and unsubtracted cDNA libraries with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA and an α-chain interleukin 2 receptor (IL2R) cDNA. The subtraction was intended to clone genes in activated T-cells. The non-differentially expressed cDNA, G3PDH, was present in the "tester" and "driver" at similar levels but was not present in the subtracted cDNA. The differentially expressed cDNA, IL2R, was enriched after subtraction by 200-fold. The subtraction with pooled strawberry fruit cDNA does not match this efficiency.

One of the major differences between the strawberry and T-cell is that fewer differentially expressed genes are likely to be present between soft and firm fruit than in non-activated and activated T-cells, particularly as the strawberry cDNAs pooled were from 20 individuals. The pooling evidently does not completely equalise the genetic background between the strawberry populations, as a putative G3PDH cDNA clone not known to be related to texture, was isolated from the subtracted firm library which showed higher transcript levels in the soft pool than in the firm pool (Fig. 4.8). If other genes not involved in determining texture showed similar differences in their transcript levels then enrichment would not be targeted specifically toward the texture related genes. The smeared appearance of the subtracted products and the absence of discrete bands (as obtained for the sample spiked with *Hae* III digested ϕX174 DNA) is evidence that the subtraction was unsuccessful. The cDNA clones putatively encoding Aconitase (FFC95), Invertase (FFC18) and flavanone-3-hydroxylase (FFC89) similarly have no obvious connection with texture and would be expected to have similar transcript levels in firm and soft fruit after pooling. Clones like the putative flavanone-3-hydroxylase are not thought to have a texture-related role in strawberry fruit as it is part of the anthocyanin pathway and its expression correlates with an increase of anthocyanin content (Manning, 1998).

Further evidence for the inefficiency of the subtraction procedure is apparent in Figure 4.6. The three known cDNAs, UDP glucosyl transferase, elongation factor I and acyl carrier protein (Manning, 1998), did show a reduction in abundance, but not the levels reported by Gurskaya *et al.*.. (1996). It appears from the evidence here and from previously published data, that the simpler the differences between two systems the more efficient the SSH technique (Chu *et al.* 1997, von Stein *et al.* 1997, Kuang *et al.* 1998, Evans and Wheeler, 1999). This is also illustrated by the success of the control reaction where viral DNA was mixed with skeletal muscle DNA and could be isolated after subtraction.

Of the 20 sequenced cDNAs, the clone most likely to be related to fruit firmness is that encoding CAD (SFC39). Lignins are not generally a constituent of fruit cell walls but could be present in the vascular bundles that run from the achenes to the calyx. These vascular bundles may anchor the achenes and provide mechanical strength to the receptacle. This clone showed higher transcript levels in the firm pool compared to the soft pool. Although these data suggests a possible role for CAD in fruit firmness further data are required. For example, the expression of CAD in individual plants within the pools could be studied and correlated with firm or soft fruit. CAD has been down regulated in tobacco plants to study the effects on lignin production. Tobacco plants with depressed CAD activity showed no change in lignin amount but there were changes in the ratio of syringyl to guaiacyl monomers (S/G) (Andreé *et al.* 1998, Yahiaoui *et al*, 1998). The main effect of genetic modification was to improve cell wall degradability.

Organic acids are important flavour components in fruit. In strawberry fruit, acid content increases during development and ripening (Woodward, 1972) and decreases in overripe fruit (Reyes *et al.* 1982). Aconitase expression in fruits has only been described in sweet lime and sour lemon. One of the two isozymes identified was found in the mitocondrial fraction, declined early in sour fruit development, but was constant in sweet lime development (Sadka *et al.* 2000). The other aconitase isozyme was detected in sour lemon towards maturation. The differences in temporal and spatial expression are thought to account for the differences in citric acid levels between the two fruits. As well as its role in the TCA cycle, an aconitase isoform was found to be active during pathogen attack in soybean (Cots and Widmer, 1999). Transcript levels of aconitase and malic enzyme are low in ripe fruit but appear to be higher in the soft pool. It is difficult to see how these transcript levels in the pools could be related to firmness or to predict how their expression relates to the control of the basic biochemical pathways in fruits. A cDNA clone for malic enzyme has been isolated from grape (Franke and Adams, 1995).

Ubiquitin-conjugating enzyme is part of the ubiquitinylation process of modifying the regulatory proteins of processes such as cell cycle control, regulation of transcription, cell differentiation, stress response and programmed cell death (von Kampen *et al.* 1996). The ubiquitin-dependent pathway requires that proteins targeted for degradation become conjugated with a chain of ubiquitin molecules that act as a signal for degradation by the 26S proteosome, a 1.5Mda multisubunit protease complex. Several ubiquitin-conjugating enzymes from plants have been isolated and their structure determined (Vierstra, 1993). Little is known about the exact function of the different ubiquitin-conjugating enzymes in plants, but it is thought that, to some extent, the function of these enzymes in plants differs from other organisms (von Kampen *et al.* 1996). Protein turnover is an essential part of plant cell physiology. Controlling metabolism can be achieved by reducing the amount of key enzymes and regulatory proteins. Protein turnover can also be part of cell development for example, by breaking down redundant proteins to supply amino acids needed to make new proteins. During strawberry development, gene expression is very dynamic with many changes from immature to ripe fruit. Manning (1994) reported over 50 mRNA changes between the immature and overripe stages. (Civello *et al.* 1996) also reported changes in polypeptides during strawberry fruit ripening. Genes which are part of the ubiquitinylation process would be expected to be strongly expressed during strawberry fruit ripening. However, proteolysis-type genes do not appear part of the ripening process as the expression of an ubiquitin-conjugating enzyme is not altered when tomato fruits are exposed to a high CO_2 concentration although high CO_2 levels did block the accumulation of ripening-related genes (Rothan *et al.* 1997).

Genes involved in anthocyanin production are well known to be expressed in some ripening fruits (e.g. Manning (1998)). They may offer examples of potential variation between fruits that is unlikely to be relevant to texture. The pooling strategy was designed to minimise representation of these genes. The putative F3H cDNA clone was slightly more highly expressed in the soft pool although the clone was isolated from the firm pool subtracted library. The SSH technique was meant to remove sequences that are

common to both the tester and driver. However, it did not achieve this. Virtual Northern analysis shows that the transcript levels for the F3H clone are very similar in both pools.

An interesting clone to be isolated is a putative QM protein homologue. QM is a human cDNA originally isolated as a transcript elevated in a non-tumeric Wilms' tumour microcell hybrid (Nguyen *et al.* 1998). QM is thought to be a putative tumour suppressor gene and has been cloned from species encompassing members of the plant, animal and fungal kingdoms (Koller *et al.* 1996, , Mills *et al.* 1999, Wiens *et al.* 1999). QM also appears to have a role in cell differentiation, cell death and regulation of growth in yeast (Koller *et al.* 1996). Research has also shown that a QM homologue in yeast is involved in a late step of the 60S ribosome sub-unit assembly (Nika *et al.* 1997). The QM homologue could play an important role in determining fruit texture during early fruit development by regulating cell number. Microscopy studies of firm and soft cultivars have shown that the firm cultivar, ITA 80-52-1 had rounder, smaller and more densely packet cells at the small green stage than the soft cultivar EM294 (Oldroyd *et al.*. unpublished).

The success of SSH is influenced by factors such as driver:tester ratio, DNA concentration and the effective non-amplification of the tester/driver hybrids. The SSH technique achieves selective amplification by only amplifying tester/tester hybrids with asymmetric adapters. The length of time for the first hybridisation could have been too short to allow full hybridisation between the sequences in the tester and driver but this should been corrected by addition of fresh driver during the second hybridisation.

Improvements can be made to the way in which the SSH technique was performed. The first would be to use equal amounts of cDNA to make up the pools. This would reduce any misrepresentation of transcript due to extracting the RNA from pooled tissue. Other modifications would be to increase the time of the initial hybridisation, from 8 hours to 12 hours, to allow improved equalisation between the abundant and less abundant

molecules. This would also improve the enrichment of the differentially expressed sequences.

If the technique can be optimised, then it does have the potential to isolate putative texture-related genes. Any alternative method would have to isolate both abundantly and rarely expressed genes which rules out DD-RT-PCR and differential screening. A possible alternative method would be cDNA-AFLP which is described in the next chapter.

Chapter 5: THE ISOLATION OF TEXTURE-RELATED CLONES FROM THE F_1 GENERATION BY cDNA-AFLP.

5.1 INTRODUCTION

There are a variety of molecular techniques available to identify and clone differentially expressed genes. Chapter 4 describes the use of the suppressive subtractive hybridisation method to isolate differentially expressed genes. Although this technique was able to isolate viral DNA from spiked skeletal muscle cDNA, it appeared to be inefficient when applied to two pooled samples of high complexity. The differences between the firm and soft fruits were unlikely to be so clear cut as this, which may have explained the inefficient subtraction. This inefficiency resulted in many false positive results that were only eliminated by northern analysis.

Another recently described method termed cDNA-AFLP (Bachem *et al.* 1996), was used to identify differentially expressed genes based on the detection of cDNA restriction fragments by PCR amplification. It can be used for DNAs of any origin or complexity and has the advantage over other RNA fingerprinting techniques such as differential display-RT-PCR that

a) PCR is performed under stringent conditions which make the technique reliable and

b) cDNA-AFLP does not preferentially amplify 3'-untranslated regions.

The AFLP reaction selectively amplifies a subset of cDNA fragments. Products generated from a range of tissues can be compared on a single gel and differences detected.

The cDNA-AFLP technique has been used to identify specifically expressed genes during potato tuber development (Bachem *et al.* 1996), parasitic genes in nematodes (Jones and Harrower, 1998), cancer genes in rats (Fukuda *et al.* 1999) and pathogenicity in *Erwinia carotorora* (Dellagi *et al.* 2000). In the study of tuber development, cDNA-AFLP was used to analyse the expression profiles of developmentally regulated genes. The expression profile of an AGPase transcript derived fragment (TDF) encoded by one of these genes was confirmed by northern analysis (Bachem *et al.* 1996). It was hoped that by applying the cDNA-AFLP technique to the firm and soft pools, differences in gene expression between the two pools could be identified.

5.2 RESULTS

5.2.1 Principle of the cDNA-AFLP method

The cDNA to be analysed is digested with two restriction enzymes, one recognising four bases (*Mse*I) and and the other six bases (*Eco*RI), (Fig 5.1). The main reason for using this combination of enzymes is to produce small fragments that are easy to amplify and that separate well on a polyacrylamide gel. In addition, the complexity of the cDNA

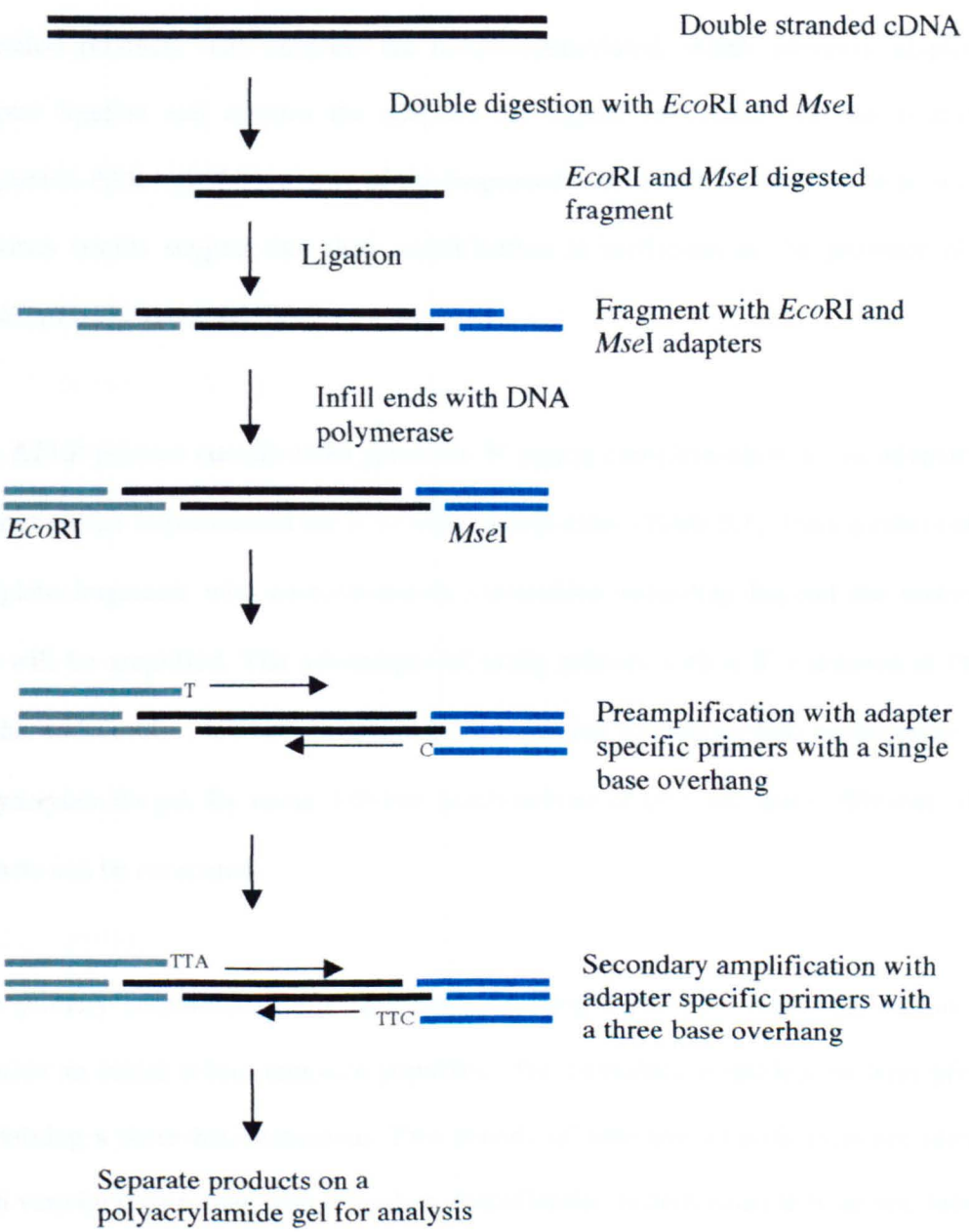

Figure 5.1 Schematic diagram of the cDNA-AFLP procedure.

fragments to be analysed on a gel is reduced by using the rare/frequent cutter combination. Adapters specific for the *Eco*RI and *Mse*I restriction sites are ligated to the digestion products. The adapters are nonphosphorylated, which prevents adapter-to-adapter ligation and ensures the adapters are ligated to virtually all the restriction fragments. Although the majority of the fragments will have *Mse*I adapters at both ends, previous results suggest that their amplification is inefficient in the presence of the *Eco*RI primer (Vos *et al.* 1995)

The AFLP primers contain three parts: the 5' region complementary to the adapter, the restriction site sequence and the 3' selective nucleotides (Table 5.1). Only a subset of the template fragments with complementary nucleotides extending beyond the restriction site will be amplified. The advantages of using primers with a 3' extension is that it further reduces the number of bands amplified making it easier to detect differences on a polyacrylamide gel. By using different combinations of primers, many different cDNA subsets can be compared.

The primary amplification with primers containing a one-base extension was used to provide an initial subset template population for secondary amplification with primers containing a three base-extension. Two rounds of selective amplification are required with very large genomes. The secondary amplification is performed with an end-labelled *Eco*RI primer to allow band visualisation. The *Eco*RI primer is used for end-labelling as the fragments required are the *Eco*RI- *Mse*I fragments. There is a small probability of amplifying *Eco*RI- *Eco*RI fragments, but as these are very rare they will not affect the

amplification. Labelling only one strand prevents the occurrence of double bands or "doublets" on the gel due to unequal mobility of the two single strands of a denatured amplified fragment.

5.2.2 Analysis of cDNA-AFLP bands by sequencing and northern analysis

The cDNA-AFLP analysis was performed on the firm and soft pools using *Eco*RI-T and *Mse*I-C primers for the primary amplification and *Eco*RI-TTA and *Mse*I-CTT primers for the secondary amplification. Between 40 and 50 bands were resolved for each sample (Fig.5.2). Some bands were present in both firm and soft fruit at similar intensities, other bands common to both had different intensities while many were present in one of the pools and absent in the other. Bands which were present in one of the samples but not the other were excised from the dried gel, reamplified and cloned into the pPCR-Script Amp SK(+) vector.

5.2.2.1 Verification of band identity

One of the difficulties encountered with the cDNA-AFLP technique is how to verify that the fragment isolated and cloned from the gel is the same as the visualised band in the original amplification. Bands could contain fragments of lower abundance with the same mobility and have *EcoRI*/*Mse*I ends and the band of interest. This was achieved by selective PCR amplification and confirmed by sequencing the DNA fragment. The fragment was eluted from the gel into an elution buffer. The elution buffer was cleaned

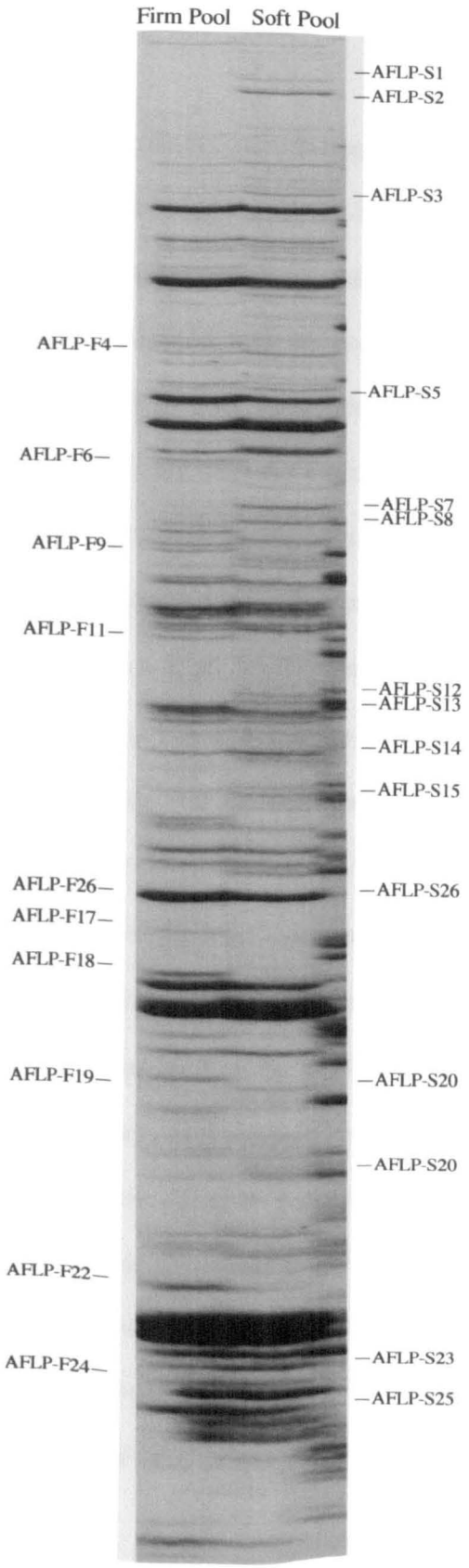

Figure 5.2 cDNA-AFLP fingerprint of soft and firm pools using the primer combinations of *Eco*RI-T/*Mse*I-C and *Eco*RI-TTA/*Mse*I-CTT. The fragments were separated on a 6% polyacrylamide gel. The size range of spearated fragments is 50-600 nucleotides. Bands sampled for cloning are indicated on the appropiate size.

by a spin column to remove any polyacrylamide fragments and filter paper and used as a template for PCR amplification using the *Eco*RI-TTA and *Mse*I-CTT primers. This should have resulted in the target fragment being preferentially amplified. The amplified fragment was cloned and transformed into *E. coli*. For all amplified and cloned fragments, two independent transformants were screened by two PCR reactions. The first reaction used the *Eco*RI-TTA and *Mse*I-CTT primers and the second used only the *Mse*I-CTT primer. Only colonies which produced a PCR product in the first reaction were taken forward for further analysis. If a fragment was amplified with the *Mse*I primer alone then it indicates that a *Mse*I- *Mse*I fragment had been cloned from the gel. Two independent transformants per cloned band were selected for sequencing. This enabled confirmation that the insert was derived from an *Eco*RI-*Mse*I fragment in the original gel and if identical sequences were obtained then this would be strong evidence that the fragment was cloned from the band visualised on the gel. Another method to verify the correct fragments had been cloned is to compare the expression profile of the corresponding gene with that of the band on the gel (Bachem *et al.* 1996). This method is not suitable for this study as only two samples are being compared which represent pools of twenty individuals with different genotypes. If the visualised band was polymorphic then northern analysis would not show the same expression pattern as the cDNA-AFLP gel. DNA cloned from twelve of the nineteen bands produced independent transformants having identical inserts while seven of the nineteen had different inserts. All inserts analysed had *Eco*RI and *Mse*I primer sequences.

5.2.3 Characterization of the cDNA-AFLP fragments

Sequence homology searches were carried out using the BLASTX search facility available at the NCBI web page. Eight of the 26 clones sequenced from the nineteen fragments showed homology to sequences in the database (Table 5.1). The sequence AFLP-S1 had a 57% amino acid identity to a hypersensitivity-related cytochrome P450 (T03275) from *Nicotiana tabacum* and to another similar sequence (T03634) also from *N. tabacum*. Northern analysis did not detect any transcript after a 17-day exposure in 10μg of total RNA from either the firm or soft pools. The cDNA-AFLP method may be able to detect very rare transcripts by virtue of the amplification achieved with the PCR although band intensity on the gel may not be strictly quantitative. This could be one explanation why no mRNA transcript was dected by northern analysis.

The two fragments cloned from the firm-specific band AFLP-F6 were not identical. AFLP-F6A had a 71% amino acid identity to a hypothetical protein predicted from the sequence of BAC F24J5 (AAD49983) from *Arabidopsis thaliana*. Similar identities were found with an unknown protein (AAD32904) from chromosome 2 of *A. thaliana* and another hypothetical protein from BAC F15H18 (AAF25997) from *A. thaliana*. Northern analysis of the AFLP-F6A clone showed low but similar transcript levels in both the firm and soft pools (Fig. 5.3). The second fragment, AFLP-F6B, cloned from the band had 88% amino acid identity to an isocitrate dehydrogenase clone (AAD51361)

Table 5.2 Characterisation of clones from the firm and soft cDNA-AFLP fingerprints. Homology is based on amino acid identities with the length of translated sequences in brackets. See appendices VIII and IX for sequences and alignment.

Clone	Clone size (bp)	Putative identity	Related sequence and accession number	% amino acid identity (length)
Firm specific				
AFLP-F6A	330	Hypothetical protein from BAC F24J5	*Arabidopsis thaliana* (AAD49983)	71 (109)
AFLP-F6B	331	Isocitrate dehydrogenase	*Citrus limon* (AAD51361)	88 (80)
AFLP-F9A	298	Ribosomal protein L32	*Arabidopsis thaliana* (T1480)	86 (46)
AFLP-F9B	301	no significant homology		
AFLP-F16	200	no significant homology		
AFLP-F18	163	no significant homology		
AFLP-F19	144	no significant homology		
AFLP-F24	73	no significant homology		
AFLP-F26B	180	no significant homology		
AFLP-F26D	179	no significant homology		
Soft specific				
AFLP-S1		Cytochrome P450	*Nicotiana tabacum* (T03275)	57 (187)
AFLP-S7	310	Isocitrate dehydrogenase	*Citrus limon* (AAD51361)	88 (71)
AFLP-S8	303	no significant homology		
AFLP-S12	235	Hypothetical protein F17M5.10	*Arabidopsis thaliana* (T05976)	64 (64)
AFLP-S13	231	no significant homology		
AFLP-S14A	211	Protein kinase endoribonuclease	*Arabidopsis thaliana* (AAD32909)	75 (68)
AFLP-S14B	212	Phytochelatin synthase	*Arabidopsis thaliana* (CAA07251)	54 (62)
AFLP-S15	207	no significant homology		
AFLP-S20A	143	no significant homology		
AFLP-S20B	142	no significant homology		
AFLP-S21A	130	no significant homology		
AFLP-S21C	133	no significant homology		
AFLP-S22B	108	no significant homology		
AFLP-S22C	103	no significant homology		
AFLP-S25	63	no significant homology		
AFLP-S26	182	no significant homology		

F Firm
S Soft
A, B, C, D Independent transformants from cloned fragments

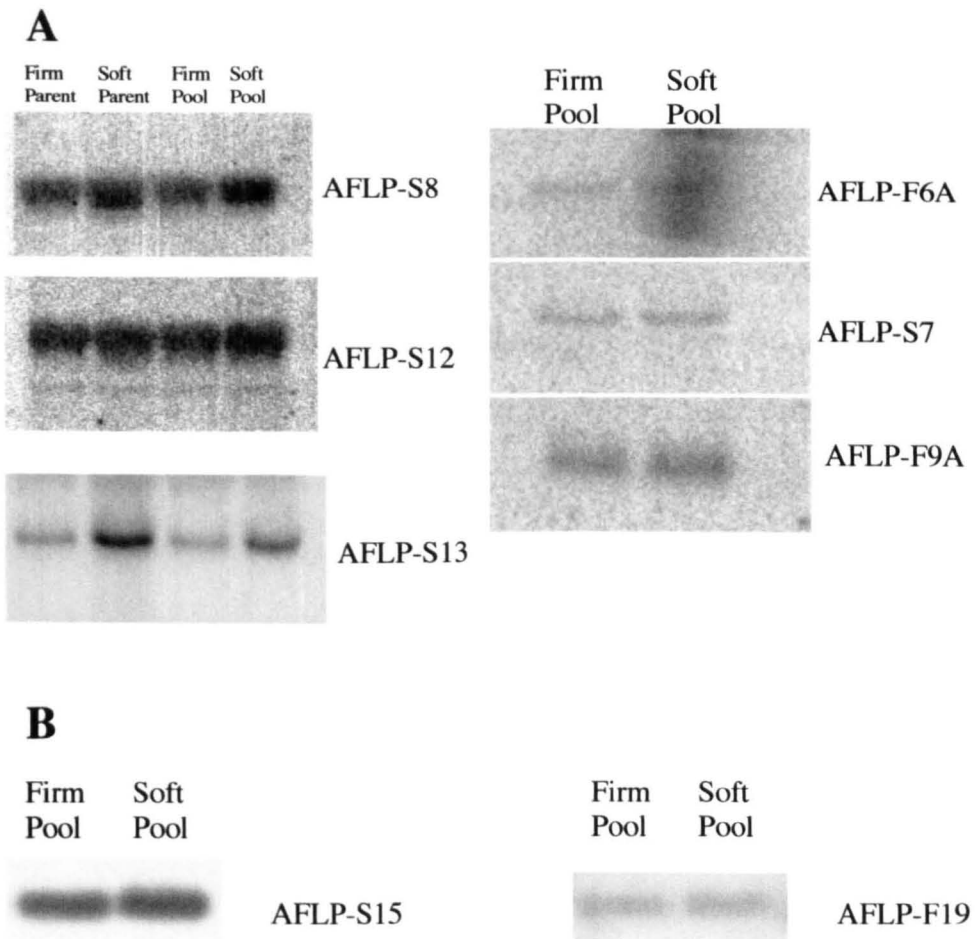

Figure 5.3 Analysis of gene expression in firm and soft pools and fruit from parental plants. **A.** northern-blot analysis of selected clones obtained from the cDNA-AFLP. Total RNA (10μg per lane) was fractionated by denaturing agarose gel electrophoresis, blotted on to nylon membranes, and hybridised with radiolabeled cDNA probes (indicated on the right of each panel). **B.** Virtual northern analysis of two cDNA-AFLP clones. cDNA from both firm and soft pools was amplified using *Mse*I-core and *Eco*RI-core primers for 25 cycles. The PCR products were fractionated by agarose gel electrophoresis, blotted to nylon membranes, and hybridised with radiolabeled cDNA probes indicated to the right of each panel.

from *Citrus limon*. Other closely related sequences were from *Solanum tuberosum* (T07402) and *Apium graveolens* (CAA73139) (celery).

Two clones from AFLP-S7 (AFLP-S7A and AFLP-S7B) had inserts and were 88% homologous at the amino acid level to an isocitrate dehydrogenase clone (AAD51361) from *Citrus limon*. These clones share identity to AAD51361 with the clone AFLP-F6B. The inserts were cloned from bands amplified from the different pools and with significant different sizes of 331bp (AFLP-F6B) and 310bp (AFLP-S7) so contamination during band excision was unlikely. The clones, AFLP-F6B and AFLP-S7, had identical nucleotide sequences along a 239pb overlap. Northern analysis of AFLP-S7 shows low levels of transcript in the firm and soft pools after a 17-day exposure (Fig. 5.3A) but no convincing difference in signal intensity between the firm and soft pools.

Fragments cloned from the AFLP-F9 band were not identical. AFLP-F9A had 86% amino acid identity to ribosomal protein L32 (T1480) from *A. thaliana* and a 60S ribosomal protein (P5142) from *Zea mays*. Northern analysis indicated that higher levels of this transcript were present in the soft pool than the firm pool (Fig. 5.3A). The second fragment that was cloned and sequenced from the same band did not show any homology to sequences in the database at the time of writing. The two fragments cloned from the AFLP-S12 band were identical and had 64% amino acid identity to the hypothetical protein F17M5.10 (T05976) from *A. thaliana*.

The cloned cDNA isolated from the AFLP-S14 band were not identical. The fragment AFLP-S14A had 75% identity at the amino acid level to a putative protein kinase/endoribonuclease (AAD32909) from *A. thaliana*. The second fragment, AFLP-S14B had 54% amino acid identity to a putative phytochelatin synthase (CAA7251) from *A. thaliana*.

None of the other sequences obtained from the cloned cDNA-AFLP bands had significant homology to sequences in the NR database. Those sequences were also compared with sequences in the EST databases using BALST. None of the clones showed strong homology with any EST sequence from the database. A virtual northern of AFLP-S15 (Fig 5.3B) showed a strong overall signal with a higher transcript level in the soft pool than the firm pool. The AFLP-F19 probe hybridised weakly on the virtual northern (Fig 5.3B) to both pools with no detectable differences in transcript levels.

To improve the detection of transcripts in northern analysis, RNA probes were prepared from PCR amplified plasmid inserts using the M13 forward and reverse primers and RNA polymerase. For RNA probes, the anti-sense strand has to be synthesised. The AFLP fragments were cloned into plasmids by blunt ending making it difficult to predict the orientation of the inserts and hence whether to use T3 or T7 RNA polymerase to synthesise the anti-sense strand. One RNA polymerase was selected and if this failed to produce a probe that hybridised to a complementary transcript then the other RNA polymerase was used.

The AFLP-S8 probe showed stong hybridisation to RNA from in both parents and both pools (Fig. 5.8). RNA from both parents was used to determine if probe hybridisation correlated with firm and soft pools. The AFLP-S12 probe showed a similar pattern of hybridisation but with an additional band below the main band but after washing the nylon filter under more stringent conditions (0.05% SSC, 0.1% SDS) most of the non-specific binding was removed without decreasing the signal of the lower band (Fig. 5.3A). The AFLP-S13 probe also hybridised to two bands, the second being much more pronounced. The major band hybridising with AFLP-S12 and S13 RNA probe may represent non-specific binding to one of the ribosomal bands. There were significantly higher hybridisation signals with the AFLP-S13 probe in the soft parent than the firm parent and this was repeated with the pooled samples although the overall hybridisation signal was lower (Fig. 5.3A).

5.2.4 Further characterisation of AFLP-S13 expression

The clone, AFLP-S13 was choosen for further characterisation as there were significant differences in hybridastion signals between the soft and firm pools and this was matched by the parent samples. Figure 5.4 shows the spatial and temporal expression of AFLP-S13 in developing strawberry fruit. The AFLP-S13 clone hybridised to a 2.1kb transcript. The expression of AFLP-S13 clone is not fruit specific with highest expression in the petioles. Expression levels are also high in flowers and leaf tissue but lower in the roots. The gene is expressed early in fruit development, its expression increasing during the orange stage reaching a maximum at the overripe stage. This gene

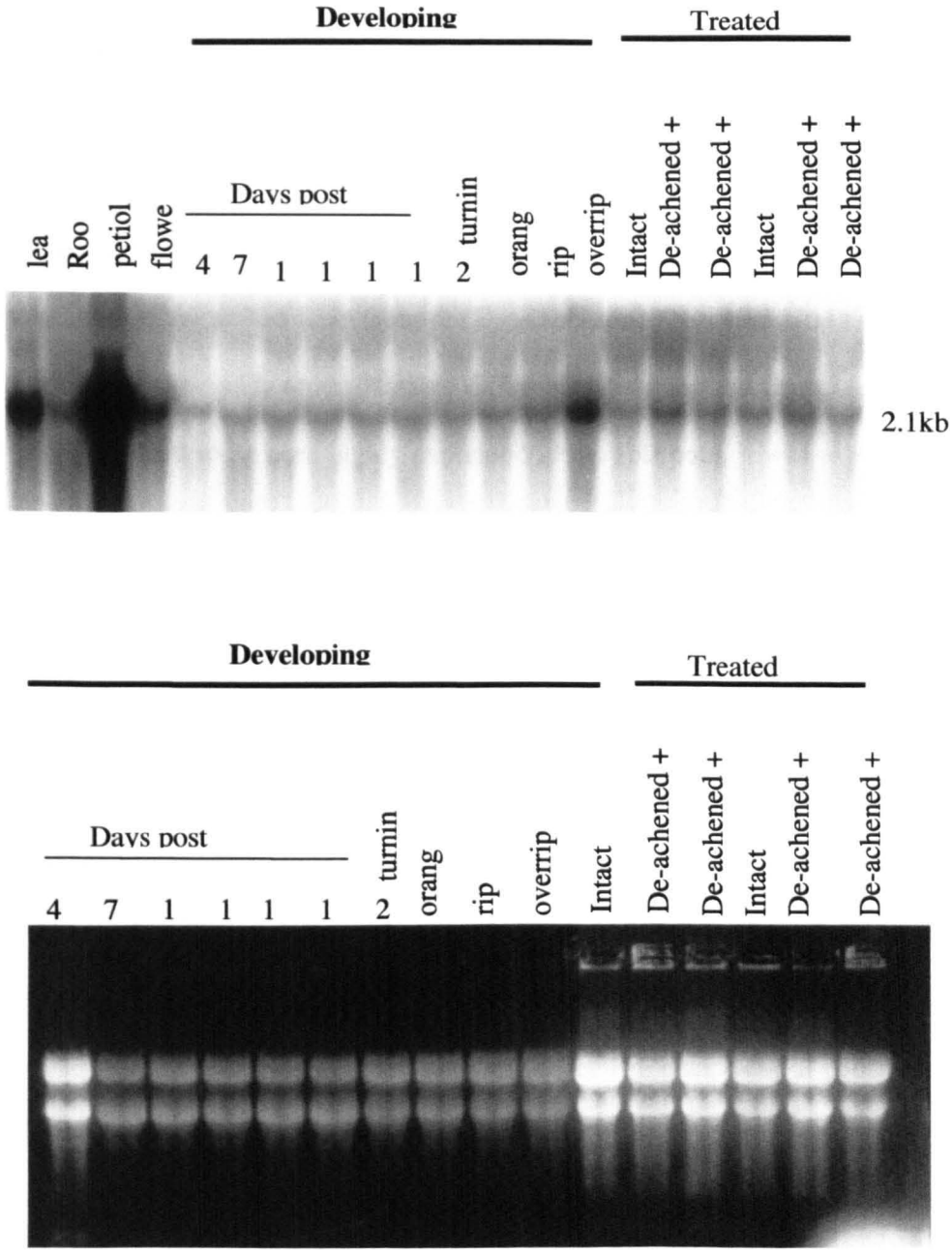

Figure 5.4 Northern blot of the expression of AFLP-S13 in strawberry in developing fruits (day 4 to overripe), leaves, petioles, flowers and roots, and in de-achened receptacles treated with auxin. Each lane contained 10 μg total RNA. Lower panel: total RNA from stained with ethidium

is under auxin control as the deachened water treated fruit have a higher expression than fruit treated with auxin analogue NAA.

The expression of AFLP-S13 was also investigated in ripe fruits from two of the firmest lines and two of the softest lines from the F_1 generation. The expression levels in these fruits were very low with no discernable differences between the fruits. As the AFLP-S13 gene appeared to be nore highly expressed in the soft pool, it was expected that its expression in the softest fruit in this pool would be higher than in the firm fruit.

To examine further if the expression of AFLP-S13 was correlated with fruit firmness fruits from strawberry plants that had a range of firmness were obtained from the East Malling breeding programme. The cultivars EMR154, EMR110 and Selva were assessed from previous sensory testing to have very firm fruit, cultivars EMR132 and Bolero were classified as firm, cultivars Evita and Mara des bois were classed as intermediate and cultivar Tango was assessed as producing soft fruit (Simpson, D. personal communication). The harvested fruit were tested for firmness using a penetrometer as described in chapter 2. The firmness of the harvested fruit did correlate with the sensory results but the differences in firmness between the intermediate fruit. Evita and Mara des bois, and the soft fruit, Tango, were small (Fig 5.5). The firmest fruit of EMR154, were >2-fold firmer than the softest fruit of Tango. These initial results suggest that sensory assessment can accurately distinguish differences in fruit firmness, but that there is probably some overlap between cultivars.

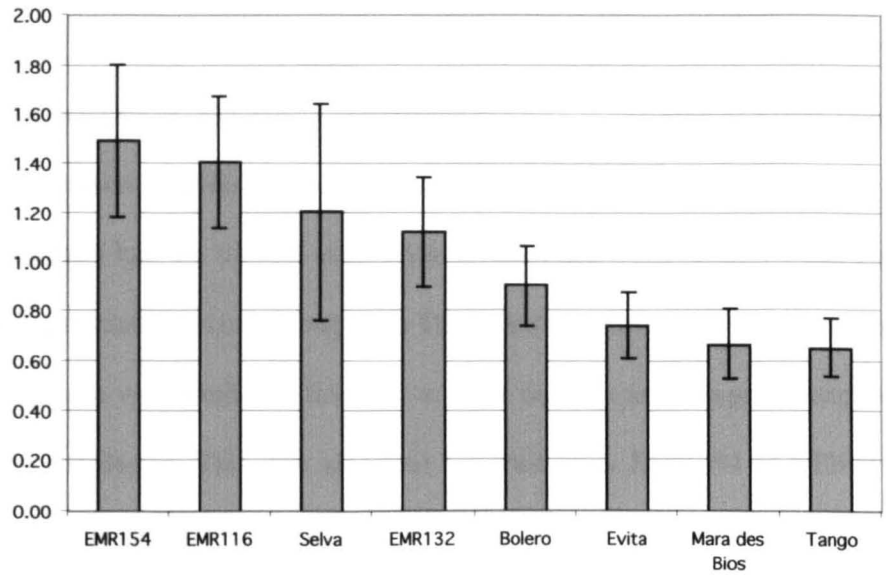

Figure 5.5 Firmness of selected cultivars from the East Malling breeding programme. Vertical bars indicate standard deviation.

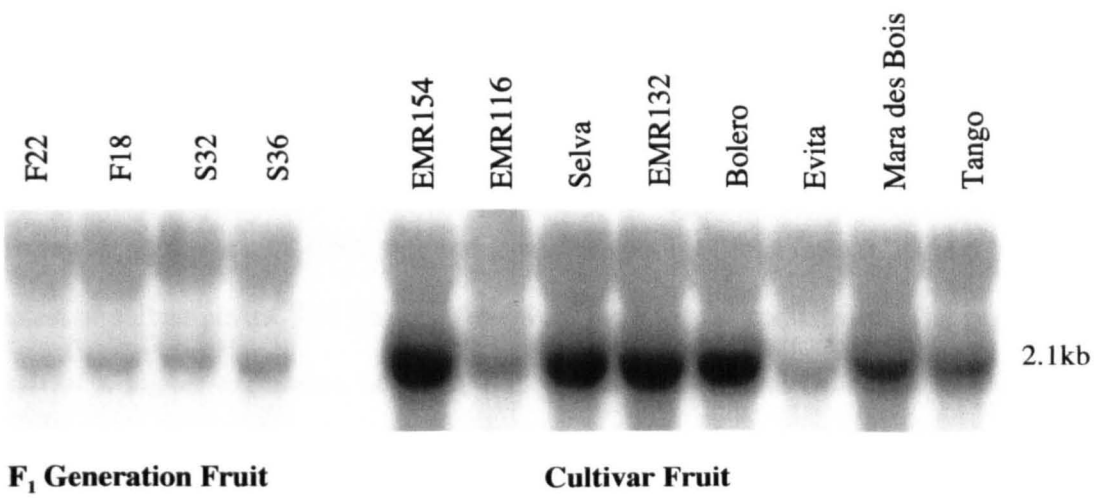

Figure 5.6 Northern-blot analysis of AFLP-S13 expression in ripe fruit from selected F_1 plants and a range of strawberry cultivars. Total RNA (10μg per lane) was fractionated by denaturing agarose gel electrophoresis, blotted to nylon membranes, and hybridised with the radiolabeled cDNA probe.

Northern analysis of AFLP-S13 expression in fruits of selected cultivars showed widely different transcript levels (Fig. 5.6). Fruits from EMR154, Selva, EMR132, Bolero and Mara des bois had the highest expression levels. Fruit from EMR116 and Evita had the lowest expression levels and fruit from Tango had moderate expression levels. Although Evita fruit was very similar in firmness to Mara des bois and Tango the expression levels were very different. This was also true for fruit from EMR154 and EMR116, which produced similar penetrometer readings but very different expression levels. The expression of AFLP-S13 did not correlate with fruit firmness between cultivar fruits. If AFLP-S13 was an important determinant of fruit firmness as first indicated by the transcript levels in the parent plants then its expression would be expected to be higher in softer fruit.

The transcript levels in the firm and soft pools were analysed further by northern blotting using the clone AFLP-S13 as a probe. To assess how transcript levels were affected by pool size total RNA was extracted from pools of fruit from 5, 10, and twenty firmest or softest plants. The parental fruit were included for comparison. The expected result of a higher expression of AFLP-S13 in the soft parental fruit than in the firm parental fruit was obtained (Fig 5.7). Transcript was detected in the pools of five and 20 individuals but not in the pools of ten individuals. This was surprising, as previous studies had indicated that the RNA from all pools was intact and that the RNA from the pools of ten individuals did produce ^{35}S-labelled polypeptides during the *in vitro*

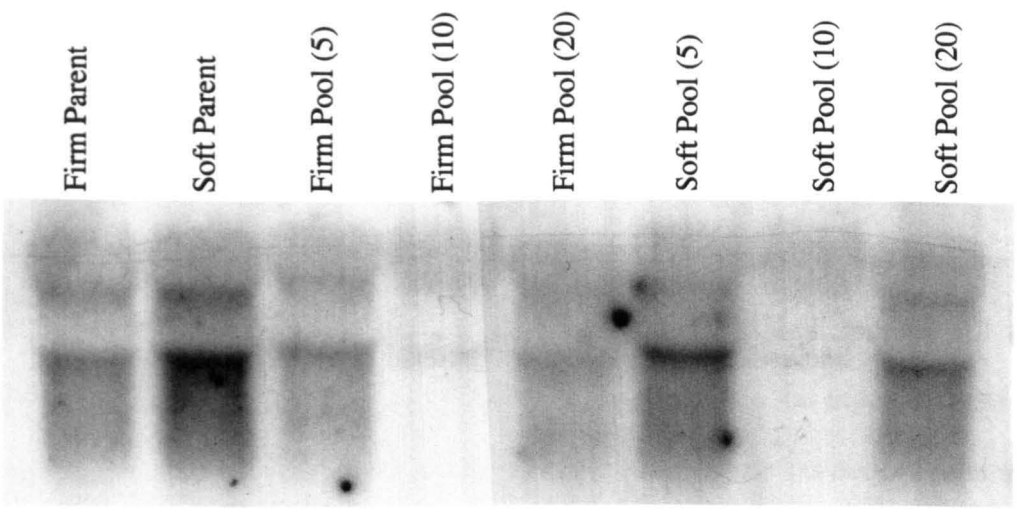

Figure 5.7 Northern-blot analysis of AFLP-S13 with parents and the three firm and soft pools. Total RNA (10μg per lane) was fractionated by denaturing agarose gel electrophoresis, blotted to nylon membranes, and hybridised with the radiolabeled cDNA probe. Numbers in brackets refer to number of individulas in a pool.

translation analysis (data not shown). The pools of ten individuals include the fruit that make up the pool of five individuals. The transcript levels in the soft pools were higher than in the corresponding firm pools reflecting the differences between the parental plants. What is interesting is that in both the firm and soft pools, the AFLP-S13 transcript was more abundant in the pools of five individuals than in the pools of 20 individuals. This indicates that as the number of individuals of a pool increases there is a dilution effect with respect to the AFLP-S13 clone.

5.3 DISCUSSION

Using just one set of AFLP primers having a three-base extension it was possible to visualise over 40 band differences between the firm and soft pool samples. All the clones isolated from the cDNA-AFLP gel and sequenced showed the expected terminal sequences, i.e. the restriction site and the three base extension residues. There was perfect matching between the primers and substrate, upon which the high fidelity of the cDNA-AFLP method depends. However, this did not prevent different cDNA fragments from being isolated from a single band excised from the gel. One of the major problems in isolating PCR fragments from a gel is that a band can correspond to one or several DNA fragments. Identical fragments were isolated from eleven of the excised bands whereas seven contained a mixture of fragments. Verification that the bands are genuine PCR amplification products is by sequencing and northern analysis. If sequencing shows the presence of the expected terminal sequences then northern analysis can determine whether the clone's expression can match the fingerprint profile.

Only eight of the 26 clones sequenced showed any homology to sequences in the database. It is significant that the majority of the sequences lacking homology are under 200bp in length. The most convincing matches were found when searches were carried out using the longer fragments. None of the sequenced clones share homology with genes known to be related to fruit texture.

Clone AFLP-S1 encoding cytochrome P450 is one such example. In animals, the P450 system metabolises foreign chemicals and natural compounds such as heme and steroid hormones (Elliot and Elliot, 1997). Cytochrome P450 probably has a similar role in plants, which have a high level of P450 activity. P450 has been isolated in ripening grape berries by differential screening (Davies and Robinson, 2000). It is thought that P450 and other stress induced genes are expressed in these fruits either as a result of the stress of storing high concentrations of sugars within the cell and the stress of rapid cell expansion or that they are an integral part of the ripening process. P450 has also been demonstrated to be part of the ripening defence mechanism against the fungus *Colletotrichum gloeosporioides* in ripe red pepper fruit (Oh *et al.* 1999). P450 in plants has a wide range of roles and further analysis on the putative P450 clone is needed to decipher if it is developmentally regulated or constitutively expressed.

Phytochelatins are a family of heavy metal-inducible peptides important in the detoxification of heavy metals. Phytochelatin synthase (PC synthase) has been cloned from *Arabidopsis* (Ha *et al.* 1999) and tomato (Chen *et al.* 1997). The phytochelatin

gene, CAD1, from *Arabidopsis* is activated by a variety of heavy metals but not cadmium while PC synthase activity in tomato is activated by cadmium. Interestingly it is reported that PC synthase is present in roots and stems but absent from leaves and friuts (Chen *et al.* 1997). The putative clone PC synthase from strawberry was isolated from the soft fruit pool. Along with P450, PC synthase does not have an obvious connection with fruit texture, as their major roles appear to be stress related.

The principle role of isocitrate dehydrogenase (IDH) is to catalyse the reaction of isocitrate to α-ketoglutarate in the TCA pathway. The role of IDH in tomato fruit ripening has been explored by Gallardo *et al* (1995). They reported that IDH activity declines during the breaker stage but increases during the late ripening stage when most lycopene synthesis occurs. The enzyme is probably involved in the metabolism of C-6 organic acids and in accumulation of glutamate in the ripe tissue (Gallardo *et al.* 1995). The expression of the putative IDH gene (AFLP-S7) is similar in both the firm and soft pools and with its possible role in organic acid accumulation in ripening tomato fruit it seems unlikely that IDH is involved directly in determining fruit texture.

A clone showing homology to the ribosomal protein L32 (RPL32) was isolated from the firm pool. In *Saccharomyces cerivisiae* ribosomal protein L32 influences both splicing of its own transcript and the processing of rRNA (Vilardell and Warner, 1997). Yeast mutants that are deficient in RPL32 are also deficient in 60S ribosomal units. Genes which encode proteins that have a protein kinase/ribonuclease function are reported to be part of a signal pathway that detects unfolded proteins in proximity to endoplasmic

reticulum (ER) and inhibits the up-regulation of genes encoding chaperone ER proteins (Sidrauski *et al.* 1998). Plants are likely to have proteins with similar functions, but protein folding and ribosomal processing are not predicted to have a specific effect on fruit texture.

The initial results suggested that the clone AFLP-S13 was related to texture as the transcript level in the soft pool was significantly higher than in the firm pool. Spatial and temporal analysis shows that AFLP-S13 is not fruit specific but its expression does correlate with fruit softening. It is interesting that this clone shows the highest expression in petiole tissue (Fig. 5.4). While the putative identity of AFLP-S13 is unknown at present its spatial and temporal activity does offer the possibility that it could be a gene encoding a protein which plays a part in the dismantling of cell walls that may be related to abscission zones. By analogy, the polygalacturonase genes TAPG1 and TAPG4 are abundantly expressed in abscission zones and in the pistils of mature flowers in tomato (Hong *et al.* 2000). The promoters of these genes were fused to the β-glucosidase (GUS) reporter gene and activity was detected in the abscission zones of petiole, flower and fruit pedicel, flower corolla and fruit calyx. Another possible function of AFLP-S13 is in jasmonic acid production. Jasmonic acid (JA) and its methyl ester (MeJA) have been reported to promote the abscission of bean petiole and are thought to act by changing the cell wall polysaccharide metabolism in abscission zones and increasing cellulase activity (Ueda *et al.* 1996, Miyamoto *et al.* 1997). (Gansser *et al.* 1997) have reported that two isomeric jasmonates isolated from strawberry had a maximum concentration in immature fruits and then steadily decreased during fruit

ripening. This pattern is similar to that in sweet cherry (Kondo *et al.* 2000) in which JA and MeJA concentrations were high in the pulp during early growth stages and then decreased during harvest. This pattern is also mirrored in apple fruit development. Postharvest application of (exogenous) JA and MeJA decreases fruit acidity but does not affect fruit firmness and soluble solid content in apples (Fan *et al.* 1998). Although strawberry is a nonclimacteric fruit, its JA activity appears to be similar to that of climacteric fruits. The full length cDNA clone of AFLP-S13 will need to be sequenced in order to compare database homologies more fully and predict a function in fruit ripening.

A problem with just using the parent plants and pools for expression analysis was highlighted by the clone AFLP-S13. The data appeared to be consistent in that higher transcript levels of AFLP-S13 were present in the soft parent and the soft pool but lower in both the firm parent and firm pool. However, the relationship between AFLP-S13 and fruit texture does not appear to be very strong when AFLP-S13 expression is examined in selected cultivars. What is surprising is that the expression of AFLP-S13 in the fruit of selected cultivars was much more variable than in the F_1 fruit. The F_1 fruit which were grown under glass would have been exposed to very different conditions to the cultivars from the breeding programme that were grown in the field and this could have accounted for expression differences. Environmental factors and genetic differences could have obscured the relationship between between AFLP-S13 and firmness in field grown conditions. Pooling of the F_1 fruit should have minimised the variability of the genetic background.

The role of AFLP-S13 in fruit texture is unclear. Further work to isolate a full-length cDNA clone might enable better northerns to be obtained. RT-PCR may be useful in confirming the expression of this clone. It appears from figure 5.7 that selecting the appropiate pool size is important. It might also be instructive to determine the expression of this gene in fruit from individuals within the pools.

Improvements to the cDNA-AFLP method could be considered, including using a different combination of restriction enzymes appropiate to strawberry. By analysing strawberry sequences in the databases, a restriction enzyme combination may be found which restricts the majority of sequences. This is limited in application as there are less than 200 strawberry sequences, not including ESTs in the databases whereas *Arabidopsis* and maize sequences number in the thousands. Modifications to the choice of restriction enzymes will not overcome the major disadvantage this method has with the two samples. The method will always show the polymorphic differences between the firm and soft pools. One possible alternative would be to select bands that are present in both samples but show an up/down expression profile. This would only work if the cDNA-AFLP fingerprint correlates with transcript levels. Another possible alternative would be to use a single cultivar and compare fruit at different ripening stages. This does not specifically target texture-related genes directly, but it would avoid the problem of polymorphic differences and genes that are up or down-regulated during fruit development would be targeted. If samples from several fruits with a range of firmness were displayed together then it might be possible to identify bands whose intensity

correlated with this phenotype. The cDNA-AFLP method may not be appropiate for samples of mixed genotype.

Previous studies, which have used cDNA-AFLP, have always compared samples from a single organism. Bachem *et al* (1996) used the technique to analyse the transcriptional changes during potato tuberisation. Two clones were isolated, one codes for the storage protein, patatin, and the second codes for ADP-glucose pyrophosphorylase. The cDNA-AFLP fingerprint of the two clones was comparable to expressions observed by northern analysis.

Chapter 6: EXPRESSION OF SELECTED CELL-WALL ASSOCIATED GENES IN THE FIRM AND SOFT POOLS AND PARENTS OF THE F_1 GENERATION.

6.1 INTRODUCTION

Fruit ripening is a complex biochemical event in which the fruit receptacle undergoes programmed changes resulting in the accumulation of red anthocyanin pigments, changes in the cell wall structure that cause a loss of firmness and an increase of sugars and volatile flavour compounds. Changes in fruit texture are largely due to degradation of the cell wall polysaccharides. In strawberry the changes affect the pectins and the cellulose-xyloglucan framework (Redgwell *et al.* 1997a) and there is a marked increase of polyuronide solubilisation from the cell wall (Knee *et al.* 1977). In tomato, the best studied fruit, the solubilisation is thought to occur due to hydrolytic enzymes. Polygalacturonase (PG) plays an important role in pectin depolymerisation during ripening in tomato, although PG is not detectable in strawberry (Barnes and Pratchett, 1976). The method of solubilisation of polyuronides is thought to be different in strawberry as there is no reduction in pectin chain length during softening (Huber, 1984). However, in strawberry, polymers in the hemicellulose fraction change from high to low M_r polymers during ripening (Huber, 1984). The depolymerisation does correlate with increased activity of cellulase in strawberry (Barnes and Pratchett, 1976) and in other fruits such as avocado (Pesis, 1978) and pepper (Harpster *et al.* 1997).

Cellulase (endo-1,4-β-D-glucanase) appears to be a strong candidate for fruit softening in strawberry. Manning (1998) reported the cloning of a cDNA from strawberry fruit, showing homology to cellulase that showed expression in a ripening related and fruit specific manner under the control of auxin. This was also reported later by (Harpster *et al.* 1998) who showed that auxin applied to white deachened fruit repressed accumulation of cellulase mRNA. Recently, cellulase (*cel*1) was down regulated in strawberry fruit, which suppressed mRNA accumulation but did not affect fruit firmness (Woolley *et al*, personal communication). A second strawberry EGase gene has been identified (*cel*2) and is expressed in vegetative tissue such as leaves, stolons and young plants (Trainotti *et al.* 1999) and green fruit (Llop-Tous *et al.* 1999, Trainotti *et al.* 1999). Expression of *cel*2 in tomato fruit was suppressed but this did not affect fruit softening (Brummell *et al.* 1999). However, the force required in breaking pedical abscission zones increased indicating that the Cel2 gene contributes to cell wall disassembly in fruit abscission zones (Brummell *et al.* 1999).

Another class of protein that may be involved in cell wall changes during ripening is the expansins. These are proteins which have been demonstrated to induce cell wall extension *in vitro* (McQueen-Mason *et al.* 1992). Recently, expansins isolated from strawberry fruit have been shown to induce cell wall extension *in vitro* (Harrison *et al*, personal communication). Expansins do not appear to have hydrolytic activity as their loosening effect on the cell wall is reversible and does not increase over time. It is thought that expansin molecules bind to the surface of cellulose fibres and disrupt the hydrogen bonding with xyloglucan molecules. Expansin molecules have been isolated

from a wide range of tissue including *Arabidopsis* (Shcherban *et al.* 1995), rice internodes (Cho and Kende, 1997), tomato meristems (Fleming *et al.* 1997), cotton fibres (Shimizu *et al.* 1997) and strawberry fruit (Harrison, personal communication). The expression of *cel*1, *cel*2 and ripening-related expansin, potentially candidates regulating softening, will be examined in fruit from firm and soft pools and parent plants of the F_1 segregating population.

6.2 RESULTS

The expressions of the EGases have not been studied in relation to fruit firmness differences between cultivars. A *Cel*1 cDNA clone and *Cel*2 cDNA clone were provided by Ken Manning and Lindsey Woolley of HRI, respectively. A cDNA clone for expansin isolated from ripening strawberry fruit at the orange stage was provided by Dr Elizabeth Harrison of HRI. This clone was selected as it can be used as a probe for the ripening-enhanced expansin gene.

The *cel*1 probe labelled with ^{32}P by random primer labelling hybridised strongly to each lane (Figure 6.1). The transcript levels in RNA from the parents are higher than in the RNA from the firm and soft pools. The firm parent (cv ITA 80-52-1) has a higher transcript level than the soft parent (cv Tamella). The expression of *cel*1 in the parental

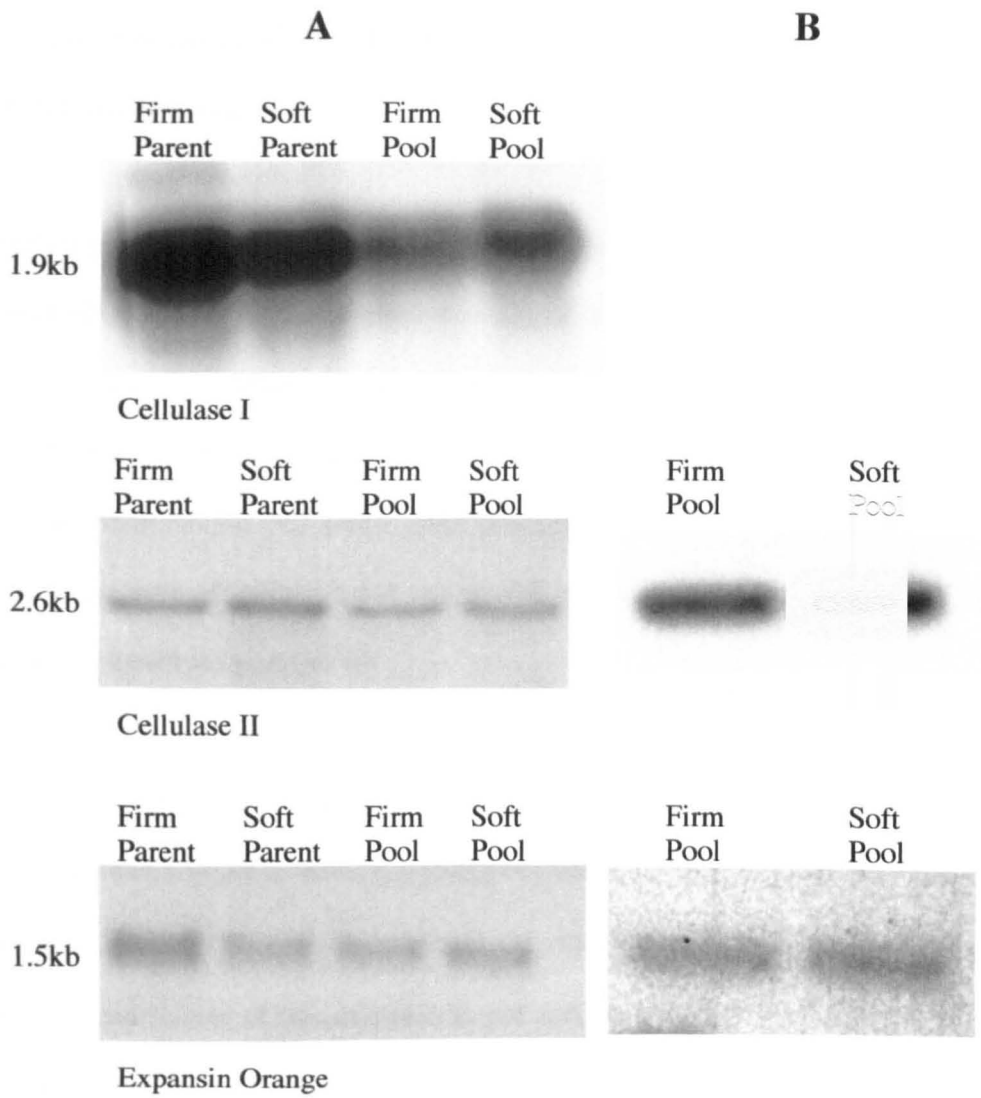

Figure 6.1 A: Northern-blot analysis of the expression of *cel*1, *cel*2 and a ripening-enhanced expansin from strawberry fruit. Each lane contained 10μg total RNA. B: Virtual northern of *cel*2 and a ripening-enhanced expansin using *Eco*RI/*Mse*I digested firm and soft pool cDNA amplified with the core *Eco*RI and *Mse*I primers.

fruit is not mirrored in the pooled fruit as the soft pool has slightly higher transcript levels than the firm pool.

The virtual northern probed with ^{32}P labelled *cel2* showed higher transcript levels in the soft pool (Figure 6.1). In the northern blot this difference was less obvious. The soft parent (cv Tamella) had higher transcript levels of *Cel2* than the firm parent (cv ITA 80-52-1). The overall signal from a 24-hour exposure detected with a phosphor-imager was much lower than for the *cel1* probe. This confirms earlier results which have reported that the expression of *cel2* is much lower than that of *cel1* (Llop-Tous *et al.* 1999, Woolley, personal communication).

An expansin cDNA clone that had been isolated from strawberry fruit at the orange stage was used as a probe to detect a ripening enhanced expansion cDNA, the main form present in ripe fruit. The virtual northern of the expansin orange clone showed slightly higher transcript levels of this expansin in the soft pool than in the firm pool (Figure 6.1). The northern blot also shows slightly higher levels of this transcript in the soft pool than in the firm pool. However, this expansin appeared to be more highly expressed in the firm parent (ITA 80-52-1) than in the soft parent (Tamella). This pattern contrasts with that of the pools, but the overall expression pattern is similar to that of *cel1* expression.

6.3 DISCUSSION

The expression profiles of EGases have been studied in a number of ripening fruits including strawberry and tomato. The strawberry *Cel1* cDNA has high homology to tomato *Cel2* cDNA, Arabidopsis *Cel1* and pepper *Cel3* (Manning, 1998). Arabidopsis *Cel1* is expressed in expanding vegetative tissues (Shani *et al.* 1997) and pepper *Cel3* in expressed in abscising zones (Ferrarese *et al.* 1995). The strawberry *Cel1* clone is fruit specific and is expressed from the turning stage onwards (Woolley *et al*, personal communication). The expression of *Cel1* is controlled by auxin produced in the achenes (Harpster *et al.* 1998). Fruit growth in strawberry requires auxin but auxin also inhibits ripening and expression of ripening related genes (Manning, 1994).

The activity of EGases in strawberry has been shown to increase during ripening (Abeles and Takeda, 1990) and this correlates with fruit softening and the increased expression of *Cel1* and *Cel2* (Woolley *et al*, personal communication). The role of *Cel1* in strawberry has been investigated by down-regulating *Cel1*. Fruit from transgenic plants that had suppressed *Cel1* transcript levels exhibited little difference in firmness from control plants (Woolley *et al*, personal communication). This would indicate that *Cel1* is not solely responsible for fruit softening in strawberry. Similar results have been reported in tomato with no effect on fruit softening when *Cel2* was suppressed (Brummell *et al.* 1999). However, the suppression of *Cel2* in tomato increased the force required to cause breakage in pedicel abscission zones. In tomato, the non-softening rin mutant lacks both *Cel1* and *Cel2* mRNA. Supplying ethylene evoked accumulation of

*Cel*1 mRNA but not *Cel2* mRNA, but did not affect texture (Gonzalez-Bosch *et al.* 1996). However, rin mutants also lack other possible cell-wall degrading enzymes and this may have prevented the restoration of softening.

Modification of the plant primary cell wall is required for both cell expansion and for developmental events, such as fruit softening where wall loosening and cell separation are important features. Recent studies suggest that the cellulose- xyloglucan network is targeted by similar enzymatic activities in both expanding cells and ripening fruit but that unique isoforms are expressed in each process. Disassembly of this structural network probably involves the concerted and synergistic action of suites of these enzyme families, where one family of cell wall modifying proteins might mediate the activity of another, providing the basis for orchestrating ordered cell wall restructuring and turnover.

The results of the analysis of expansin is inconclusive as the expression profile of the parents is not replicated by the pooled samples. This was also seen with *cel1* and *cel2*. One explanation could be that the RNA extraction process was less efficient when fruit from 20 plants were pooled together. The assumption of pooling before extraction is that an equal amount of RNA is extracted from each individual sample within the pool. If one or more samples release less RNA than other samples then the pool will be biased towards those individuals which release more RNA. The ideal method of expression analysis would include the parents, the pools, the individuals from the two pools and a

number of cultivars that show a range of fruit firmness. This would give much more information as to whether a particular clone was texture-related or not.

Expansins are likely to be involved in the ripening process and may be important in modifying the fruit cell walls enabling the cell-wall degrading enzyme to act on their substrates (McQueen-Mason *et al.* 1992). In strawberry, six expansin cDNAs have been isolated from ripening fruit (Harrison *et al*, personal communication). The most abundant expansin transcripts are formed during the late stages of development when anthocyanin production begins and the fruit start to ripen. The role of individual expansins in cell wall modification during development remains to be elucidated.

Chapter 7: FINAL DISCUSSION

The results from the analysis of the F_1 population agree with that of (Mori, 2000) that frequency distribution for firmness exhibited a normal distribution and that the trait seems to be polygenetically inherited. Mori (2000) also reported that all statistical analysis showed that firmness was a highly inherited trait under moderate genetic control. Both Mori (2000) and Shaw *et al.* (1987) report very similar heritabilities. A quantitative trait like firmness is probably controlled by multiple QTLs and plants which share the same phenotype may have different genotypes. Plants which show the same genotype can show different phenotypes when grown under different environments (Doebley, 2000). This makes inferring the genotype from the phenotype extremely difficult unless results are determined under different conditions and variation taken into account.

The cross between the two parental lines resulted in plants producing fruit with a wide range of firmness values. The data was unable to determine whether the firmness differences were ripening related or whether there were differences in cell wall synthesis during fruit development. This could have been investigated by biochemical analysis of cell wall composition of the mature green and ripe fruit from the F_1 progeny used to make up the two pools. This analysis would help to determine whether there was a biochemical basis to the differences between soft and firm fruits. This is important as the arrangement and interaction of cell wall polymers (as matrix polysaccharides or complex

cellulose microfibrils) both physically and chemically largely determine the mechanical properties of the cell wall.

The texture properties of fruits not only depend on the cell wall, but also cell to cell adhesion. Cells are joined to one another at the middle lamella and the extent and strength of adhesion will vary. Cells and intercellular spaces (air- or water-filled) are arranged into tissues, whose shape and arrangement also affect texture. The fact that strawberry plants do not produce true "fruits" could also have affect on texture due differences in tissue properties.

The poor results of the *in-vitro* translations did not allow a proper assessment of the pooling strategy. It was hoped that by comparing the firm and soft pools it would be possible to determine which would be the best pool to use for molecular characterisation. This was important, as it was not known how many individuals were required for each pool to enable the texture-related characters to stand out from the variable genetic background. Using too many individuals within a pool could dilute the texture-related characters, while too few individuals would not be able to reduce the variability of the genetic background.

The SSH did not perform efficiently with the firm and soft pool samples. This was probably because the technique was not sensitive enough to distinguish the differences between the two complex samples. Some of the clones isolated from the firm pool encoded transcripts with higher levels in the soft pool and likewise some of the soft

clones encoded mRNAs with higher transcript levels in the firm pool. Using only the two pools for transcript assessment was also insufficient. An ideal set-up would have been to assess the clones with the two pools, the parents, individuals from both pools, and a selection of commercial cultivars showing a range of firmness. Assessing the clones with all the individuals from both pools would be useful, as it would show any variable expression and give an idea of the genetic variability within the pools.

Further analysis of the parental plants would have been useful such as biochemical analysis of cell wall composition. However just using the parental plants to investigate the molecular basis of fruit texture would have been difficult, as it would be very hard to target the texture related differences from other numerous differences. The parental plants are useful as controls for checking expression of differentially expressed genes.

Northern analysis highlighted difficulties in using the cDNA-AFLP technique to identify differentially expressed genes. All the bands that were selected for further analysis were either present in the firm pool and absent in the soft pool or vice versa. Northern analysis showed that for all the clones examined, transcripts were present in both the firm and soft pools, thus contradicting their fingerprint profile. The cDNA-AFLP method is a modification of AFLP, which is a quantitative method, designed to detect polymorphic differences between samples using restriction enzymes. Each pool that was compared contained fruits from 20 individual plants each with a different genotype. Therefore, it is quite understandable that many of the differences visualised would be polymorphic rather than due to differentially expressed sequences. The cDNA-AFLP method is not

specifically targeted to texture related genes but to polymorphic differences between the two pools. To find texture-related clones, all bands that showed differences would need to be cloned and screened further by northern analysis. This would be equivalent to randomly selecting clones from a cDNA library for sequencing and expression analysis. The cDNA-AFLP technique may not be suitable for isolating-texture related clones from the two pooled samples as the polymorphic differences could be much more numerous than those related to texture.

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APPENDIX I: GENSTAT PROGRAM WRITTEN BY RODNEY EDMONSON, HRI, TO ANALYSE FRUIT FROM THE F_1 GENERATION.

```
job 'Strawberry firmness data analysis 1997 Ken Manning/Aram Buchanan'
units[269]
open'berry1.dat';channel=2;width=132
open'size4.dat';channel=3;width=132
read[channel=2] Plot,R1,R2,R3,R4,G1,G2,G3,G4,X,Y
read[channel=3] Plot,RX1,RY1,RZ1,RX2,RY2,RZ2,GX1,GY1,GZ1,GX2,GY2,GZ2

calc RW1=(RX1+RY1)/2
calc RW2=(RX2+RY2)/2
calc GW1=(GX1+GY1)/2
calc GW2=(GX2+GY2)/2


vari [nval=1076]\

FirmRed,FirmGreen,WidRed,WidGrn,HeighRed,HeighGrn,PlotNo,Miss,SizeRed,SizeG
rn
Factor [levels=2;values=269(1,1,2,2)] Fruits
factor [levels=269;values=(1...269)4] Plants

equate !p(RW1,RW1,RW2,RW2);WidRed
equate !p(GW1,GW1,GW2,GW2);WidGrn
equate !p(RZ1,RZ1,RZ2,RZ2);HeighRed
equate !p(GZ1,GZ1,GZ2,GZ2);HeighGrn

equate !p(R1,R2,R3,R4);FirmRed
equate !p(G1,G2,G3,G4);FirmGreen

calc logFR=log10(FirmRed)
calc logFG=log10(FirmGreen)

calc SizeRed=(2*WidRed+HeighRed)/3
calc SizeGrn=(2*WidGrn+HeighGrn)/3
delete WidRed,WidGrn,HeightRed,HeightGrn

calc miss=(G1.eq.c('*')).and.(G2.eq.c('*')).and.\
    (G3.eq.c('*')).and.(G4.eq.c('*'))
equate miss;Miss
restrict FirmGreen,logFG;condition=Miss.ne.1

covar SizeRed
```

```
block Plants/Fruits
anova[fprob=y] FirmRed;resid=r;fitted=f
graph r;f

covar SizeGrn
block Plants/Fruits
anova[fprob=y] FirmGreen;resid=r;fitted=f
graph r;f
anova[fprob=y] logFG;resid=r;fitted=f
graph r;f

restrict FirmRed;condition=Miss.ne.1
covar logFG
block Plants/Fruits
anova[fprob=y] FirmRed;resid=r;fitted=f
graph r;f

covar logFG,SizeRed
block Plants/Fruits
anova[fprob=y] FirmRed;resid=r;fitted=f
graph r;f

stop
```

APPENDIX II

Table II.1 Firmness of ripe fruit from the F_1 generation and plant position. Asterix indicates that no is data available.

	Fruit 1		Fruit 2		Plant Position	
Plant Number	sample 1 (N)	sample 2 (N)	sample 3 (N)	sample 4 (N)	X	Y
1	0.874	0.874	0.512	0.729	1	1
3	0.71	0.737	0.874	0.928	1	2
5	0.736	0.845	0.846	0.792	1	3
6	0.519	0.546	0.464	0.464	1	4
7	0.819	0.68	0.737	0.573	1	5
8	0.3	0.57	*	*	1	6
10	0.546	0.71	*	*	2	1
12	0.328	0.491	0.491	0.546	2	2
13	0.372	0.463	*	*	2	3
14	0.846	0.71	*	*	2	4
16	0.546	0.628	0.792	0.655	2	5
17	0.71	0.71	0.682	0.874	2	6
19	1.06	1.15	1.06	1.04	3	1
21	0.519	0.71	0.764	0.601	3	2
24	0.737	0.792	0.757	0.846	3	3
25	0.436	0.645	0.354	0.3	3	4
28	0.737	0.737	0.819	0.792	3	5
29	1.04	0.628	0.491	0.682	3	6
30	0.491	0.437	0.491	0.573	4	1
31	0.273	0.246	*	*	4	2
32	0.655	0.519	0.0682	0.628	4	3
33	0.464	0.546	0.546	0.409	4	4
34	0.519	0.382	0.437	0.519	4	5
35	0.546	0.792	*	*	4	6
36	0.409	0.546	0.409	0.328	5	1
37	0.328	0.519	0.136	0.246	5	2
42	0.464	0.437	0.409	0.328	5	3
43	0.464	0.437	0.409	0.491	5	4
44	0.546	0.792	0.546	0.737	5	5
45	0.792	1.25	*	*	5	6
46	0.464	0.573	0.191	0.191	6	1
47	0.409	0.355	0.519	0.573	6	2
48	0.628	0.737	0.601	0.546	6	3
50	0.437	0.519	*	*	6	4
52	0.601	0.737	0.464	0.519	6	5
53	0.655	0.464	0.901	0.792	6	6
54	0.409	0.437	0.382	0.464	7	1
55	0.546	0.519	0.519	0.573	7	2
56	0.764	0.655	0.546	0.382	7	3
57	0.573	0.628	*	*	7	4
59	0.601	0.546	0.573	0.573	7	5
60	0.573	0.546	0.573	0.682	7	6
61	0.519	0.464	0.71	0.792	8	1
62	0.737	0.737	0.546	0.573	8	2

Table II.1 cont.

64	0.491	0.546	0.409	0.355	8	3
65	0.682	0.874	0.655	0.71	8	4
70	0.682	0.71	0.764	0.737	8	5
71	0.519	0.519	*	*	8	6
72	0.3	0.437	0.3	0.328	9	1
74	1.04	0.764	1.04	0.764	9	2
75	0.792	0.655	0.464	0.519	9	3
76	0.437	0.409	0.546	0.573	9	4
82	0.846	0.628	0.682	0.655	9	5
83	0.464	0.464	0.71	0.218	9	6
84	0.573	0.655	0.546	0.546	10	1
85	0.573	0.573	*	*	10	2
86	0.273	0.437	0.382	0.3	10	3
87	0.437	0.491	0.382	0.601	10	4
92	0.628	0.464	0.655	0.546	10	5
94	0.6	0.491	0.464	0.491	10	6
97	0.437	0.464	0.409	0.382	11	1
99	0.764	0.655	0.737	0.628	11	2
100	0.382	0.409	0.519	0.437	11	3
102	0.737	0.601	*	*	11	4
103	0.682	0.955	*	*	11	5
109	0.273	0.246	0.328	0.273	11	6
111	0.983	1.47	0.601	0.71	12	1
112	0.764	0.874	0.792	0.792	12	2
113	0.601	0.737	0.519	0.628	12	3
114	0.382	0.328	0.437	0.546	12	4
117	0.71	0.764	*	*	12	5
118	0.792	0.764	0.382	0.464	12	6
119	0.518	0.518	0.328	0.382	13	1
120	0.682	0.682	0.601	0.655	13	2
121	0.792	*	0.874	0.901	13	3
122	0.49	0.545	0.874	1.04	13	4
123	0.355	0.437	0.355	0.3	13	5
124	0.437	0.437	0.437	0.409	13	6
125	0.601	0.464	*	*	14	1
126	0.464	0.682	0.682	0.682	14	2
127	0.628	0.628	0.628	0.846	14	3
128	0.519	0.792	0.601	0.874	14	4
129	0.71	0.546	0.601	0.546	14	5
130	0.164	0.164	0.437	0.573	14	6
131	0.737	0.573	*	*	15	1
132	0.737	0.846	1.06	0.955	15	2
133	0.519	0.601	0.328	0.328	15	3
134	0.764	0.764	0.519	0.519	15	4
135	0.491	0.3	0.792	0.71	15	5
136	0.546	0.546	0.546	0.546	15	6
137	0.546	0.628	0.601	0.628	16	1
138	0.355	0.464	0.519	0.3	16	2
139	0.655	0.601	0.601	0.464	16	3
144	0.655	0.573	0.655	0.655	16	4
145	0.71	0.546	*	*	16	5
146	0.71	0.437	0.792	0.546	16	6
148	0.437	0.164	0.464	0.409	17	1

Table II.1 cont.

149	0.682	0.71	0.71	0.68	17	2
151	0.464	0.601	0.846	0.655	17	3
152	0.792	0.901	0.737	0.682	17	4
153	0.655	0.682	0.737	0.628	17	5
158	0.491	0.464	0.546	0.573	17	6
159	0.273	0.273	0.71	0.573	18	1
161	0.874	0.792	0.519	0.546	18	2
163	0.764	0.71	*	*	18	3
164	0.409	0.355	0.682	0.628	18	4
165	0.601	0.601	0.3	0.3	18	5
167	0.464	0.491	0.491	0.546	18	6
169	0.792	0.846	0.874	0.601	19	1
170	0.464	0.683	0.628	0.628	19	2
173	0.355	0.491	*	*	19	3
176	0.491	0.573	*	*	19	4
178	0.601	0.409	0.546	0.601	19	5
180	0.464	0.464	*	*	19	6
181	0.491	0.519	*	*	20	1
189	0.874	0.928	0.955	1.09	20	2
191	0.682	0.601	1.09	0.764	20	3
193	0.546	0.491	*	*	20	4
199	0.573	0.519	0.874	0.655	20	5
201	0.682	0.682	0.519	0.682	20	6
202	0.546	0.437	0.71	0.573	21	1
203	0.846	0.874	0.819	0.874	21	2
204	0.819	0.819	0.382	0.464	21	3
205	0.409	0.464	0.3	0.328	21	4
207	0.682	0.682	0.792	0.737	21	5
208	0.546	0.601	0.546	0.628	21	6
209	0.546	0.655	0.491	0.573	22	1
211	0.573	0.764	0.71	0.682	22	2
212	0.601	0.601	0.519	0.737	22	3
213	0.764	0.792	0.464	0.491	22	4
214	0.464	0.464	0.573	0.601	22	5
215	0.573	0.601	*	*	22	6
217	0.409	0.437	0.382	0.464	23	1
218	0.573	0.579	0.273	0.378	23	2
224	0.792	0.764	0.573	0.6	23	3
227	1.2	1.42	1.17	1.12	23	4
230	0.71	0.71	0.628	0.71	23	5
231	0.273	0.464	*	*	23	6
236	0.409	0.464	*	*	24	1
238	0.437	0.355	*	*	24	2
239	0.601	0.737	0.846	0.764	24	3
240	0.737	0.846	0.819	0.573	24	4
242	0.792	0.928	0.682	0.601	24	5
243	0.464	0.491	0.328	0.3	24	6
247	0.519	0.682	0.464	0.355	25	1
248	0.382	0.601	0.519	0.519	25	2
251	0.355	0.519	0.246	0.628	25	3
253	0.846	0.955	*	*	25	4
255	0.409	0.409	0.328	0.3	25	5
256	0.191	0.3	0.628	0.628	25	6

Table II.1 cont.

257	0.409	0.464	0.355	0.355	26	1
258	0.792	0.955	0.819	1.064	26	2
259	0.601	0.546	*	*	26	3
261	0.491	0.628	0.464	0.519	26	4
263	0.573	0.573	0.328	0.273	26	5
264	0.628	0.737	0.764	0.655	26	6
265	0.464	0.792	0.519	0.437	27	1
266	0.355	0.382	0.546	0.464	27	2
269	0.328	0.273	0.218	0.273	27	3
270	0.382	0.464	*	*	27	4
272	0.601	0.71	*	*	27	5
273	0.464	0.655	0.382	0.491	27	6
277	0.49	0.681	0.573	0.464	27	10
279	0.437	0.546	0.464	0.437	27	9
281	0.601	0.764	0.874	0.819	27	8
282	0.655	0.655	0.573	0.437	27	7
285	0.573	0.546	*	*	26	10
286	0.409	0.464	*	*	26	9
287	0.792	0.764	0.737	0.71	26	8
288	0.846	0.682	1.12	1.09	26	7
289	0.328	0.273	0.628	0.682	25	10
290	0.382	0.546	*	*	25	9
291	0.655	0.628	0.546	0.628	25	8
292	1.23	1.17	0.71	0.846	25	7
293	0.246	0.409	0.519	0.437	24	10
297	0.601	0.573	0.682	0.71	24	9
298	0.628	0.546	0.682	0.628	24	8
300	0.464	0.573	*	*	24	7
302	0.355	0.6	0.273	0.355	23	10
303	0.519	0.519	0.409	0.328	23	9
306	0.437	0.437	0.764	0.7	23	8
307	0.546	0.573	0.846	0.792	23	7
308	0.218	0.328	0.355	0.328	22	10
309	0.273	0.3	0.382	0.273	22	9
311	0.628	0.464	0.409	0.491	22	8
312	0.682	0.546	*	*	22	7
313	0.682	0.601	0.628	0.628	21	10
316	0.682	0.519	0.6	0.546	21	9
317	0.46	0.599	0.573	0.655	21	8
319	0.846	0.792	0.682	0.601	21	7
322	0.678	0.737	0.327	0.437	21	10
323	0.546	0.573	*	*	20	9
324	0.246	0.246	0.246	0.273	20	8
325	0.409	0.409	*	*	20	7
326	0.409	0.573	0.519	0.491	19	10
328	0.273	0.382	*	*	19	9
330	0.409	0.518	0.519	0.601	19	8
331	0.519	0.437	0.846	0.573	19	7
332	0.546	0.682	0.846	0.601	18	10
333	0.546	0.437	0.573	0.573	18	9
334	1.09	1.17	0.792	0.792	18	8
335	0.573	0.6	0.764	0.628	18	7
336	0.572	0.463	0.546	0.464	17	10

Table II.1 cont.

337	0.573	0.546	0.601	0.601	17	9
342	0.6	0.655	0.245	0.273	17	8
343	0.409	0.437	*	*	17	7
344	0.682	0.737	*	*	16	10
345	0.546	0.519	0.382	0.491	16	9
346	0.71	0.928	0.792	0.846	16	8
347	1.037	0.71	0.819	0.874	16	7
350	0.682	0.901	1.06	0.928	15	10
351	0.792	0.628	0.546	0.546	15	9
352	0.682	0.519	0.464	0.437	15	8
354	0.464	0.573	*	*	15	7
355	0.409	0.437	0.655	0.573	14	10
357	0.628	0.819	*	*	14	9
360	0.246	0.273	*	*	14	8
364	1.09	1.04	0.737	0.901	14	7
365	0.628	0.792	0.601	0.628	13	10
368	0.328	0.3	0.355	0.355	13	9
369	0.437	0.437	0.381	0.792	13	8
370	0.682	0.573	0.874	0.901	13	7
372	0.437	0.409	1.01	1.12	12	10
375	0.655	0.819	0.71	0.601	12	9
377	0.546	0.737	0.573	0.601	12	8
378	0.601	0.628	0.437	0.355	12	7
380	0.573	0.491	0.682	0.955	11	10
381	0.355	0.464	0.409	0.437	11	9
383	0.655	0.628	0.71	0.846	11	8
384	0.3	0.437	0.355	0.355	11	7
385	0.464	0.628	0.655	0.573	10	10
386	0.71	0.628	0.846	0.573	10	9
388	0.601	0.573	0.874	0.792	10	8
391	0.437	0.655	0.3	0.246	10	7
393	0.628	0.6	*	*	9	10
394	0.928	1.04	0.764	0.846	9	9
395	0.71	0.819	0.764	0.71	9	8
397	0.928	1.01	0.764	0.901	9	7
399	0.382	0.491	0.519	0.546	8	10
401	0.628	0.792	0.628	0.792	8	9
403	0.464	0.436	0.546	0.546	8	8
404	0.518	0.518	0.573	0.491	8	7
405	0.901	0.874	0.682	0.628	7	10
406	1.07	0.928	0.628	0.71	7	9
407	0.491	0.846	0.491	0.3	7	8
408	0.409	0.519	0.491	0.382	7	7
409	0.437	0.573	0.579	0.519	6	10
411	0.901	0.71	0.874	0.901	6	9
412	0.409	0.464	0.546	0.491	6	8
413	0.464	0.546	0.437	0.437	6	7
414	0.792	0.901	0.601	0.573	5	10
415	0.573	0.409	1.17	0.819	5	9
417	0.819	1.23	0.546	0.682	5	8
422	0.437	0.464	*	*	5	7
423	0.491	0.409	0.437	0.491	4	10
424	0.355	0.328	0.519	0.519	4	9

Table II.1 cont.

425	0.546	0.491	0.491	0.491	4	8
426	0.601	0.491	0.601	0.792	4	7
427	0.573	0.464	0.3	0.164	3	10
429	0.764	1.23	0.409	0.437	3	9
430	0.573	0.546	0.437	0.546	3	8
431	0.3	0.355	0.409	0.519	3	7
434	0.682	0.955	0.546	0.628	2	10
438	0.355	0.437	0.409	0.519	2	9
443	0.6	0.764	0.955	1.01	2	8
445	0.71	0.76	0.901	0.901	2	7
446	0.71	0.71	*	*	1	10
447	0.437	0.601	0.655	0.464	1	9
448	0.491	0.546	0.601	0.601	1	8

APPENDIX III

Table III.1 Firmness of unripe fruit from the F_1 generation and plant position. Asterix indicates that no is data available.

	Fruit 1		Fruit 2		Plant Position	
Plant Number	sample 1 (N)	sample 2 (N)	sample 3 (N)	sample 4 (N)	X	Y
1	12.43	15.04	18.31	19.62	1	1
3	16.57	15.04	11.55	9.81	1	2
5	11.77	13.52	11.34	9.81	1	3
6	22.24	25.51	23.98	23.54	1	4
7	13.95	12.64	9.16	7.19	1	5
8	*	*	*	*	1	6
10	15.91	12.43	19.62	20.71	2	1
12	9.16	10.46	17.88	17	2	2
13	18.09	22.89	9.59	8.5	2	3
14	*	*	*	*	2	4
16	*	*	*	*	2	5
17	8.28	14.17	8.28	8.72	2	6
19	19.4	23.98	34.88	43.82	3	1
21	24.2	24.63	37.06	35.75	3	2
24	29.43	31.17	*	*	3	3
25	4.36	6.54	13.73	15.7	3	4
28	25.72	26.16	8.94	8.5	3	5
29	34.23	32.7	34.88	32.92	3	6
30	10.9	12.2	25.07	*	4	1
31	18.31	18.57	16.57	16.79	4	2
32	8.07	8.72	16.79	16.18	4	3
33	30.96	33.57	22.24	22.24	4	4
34	25.94	33.79	26.81	30.08	4	5
35	*	*	*	*	4	6
36	27.47	25.07	32.26	34.88	5	1
37	27.03	26.6	8.72	7.85	5	2
42	14.17	13.52	23.98	24.42	5	3
43	4.14	4.14	6.32	5.89	5	4
44	15.7	15.04	*	*	5	5
45	10.68	9.37	23.54	22.67	5	6
46	27.47	23.98	27.25	24.85	6	1
47	12.43	15.91	17.22	12.64	6	2
48	5.67	5.45	15.48	15.91	6	3
50	*	*	*	*	6	4
52	28.56	26.6	44.69	32.26	6	5
53	15.04	22.67	34.01	31.39	6	6
54	7.41	6.1	20.71	18.31	7	1
55	7.19	12.64	20.71	21.8	7	2
56	7.19	12.43	3.27	5.89	7	3
57	17.66	19.18	15.26	17.44	7	4
59	7.85	9.16	22.24	23.76	7	5
60	6.98	12.64	*	*	7	6
61	*	*	*	*	8	1

Table III.1 cont.

62	13.08	12.86	8.28	6.54	8	2
64	14.17	10.25	25.72	21.15	8	3
65	*	*	*	*	8	4
70	28.34	26.6	14.17	17.22	8	5
71	10.03	9.81	16.75	21.58	8	6
72	6.1	5.45	16.57	16.35	9	1
74	24.85	23.54	23.54	23.98	9	2
75	9.37	13.73	11.55	12.43	9	3
76	5.45	4.8	10.25	11.99	9	4
82	10.03	12.64	36.84	37.28	9	5
83	*	*	*	*	9	6
84	16.35	18.97	29.08	26.38	10	1
85	9.81	11.12	25.07	24.63	10	2
86	10.9	23.54	*	*	10	3
87	23.54	23.33	27.9	29.21	10	4
92	9.59	11.99	7.41	7.85	10	5
94	16.57	16.13	6.76	6.54	10	6
97	28.78	28.78	*	*	11	1
99	16.57	15.26	*	*	11	2
100	20.06	23.54	27.25	27.03	11	3
102	*	*	*	*	11	4
103	*	*	*	*	11	5
109	4.14	5.23	4.8	3.71	11	6
111	9.81	10.46	8.72	8.72	12	1
112	34.01	34.23	37.06	37.93	12	2
113	5.23	8.72	29.43	32.7	12	3
114	18.53	13.52	17.88	20.27	12	4
117	*	*	*	*	12	5
118	*	*	*	*	12	6
119	*	22.3	20.27	25.11	13	1
120	*	*	*	*	13	2
121	*	*	*	*	13	3
122	*	*	*	*	13	4
123	4.36	4.58	30.74	29.21	13	5
124	2.51	2.35	16.13	13.3	13	6
125	26.38	26.81	20.27	19.62	14	1
126	*	*	*	*	14	2
127	7.19	9.37	25.94	25.51	14	3
128	7.85	11.55	29.21	26.81	14	4
129	20.93	16.57	*	*	14	5
130	*	*	*	*	14	6
131	20.49	24.2	14.39	11.77	15	1
132	26.49	25.29	33.14	27.25	15	2
133	27.9	28.78	28.12	34.23	15	3
134	16.13	11.99	16.57	17.04	15	4
135	17.22	22.67	17.88	11.77	15	5
136	4.36	4.14	16.13	16.57	15	6
137	35.75	35.32	*	*	16	1
138	12.21	9.59	*	*	16	2
139	29.99	34.88	9.81	9.37	16	3
144	15.48	13.08	20.49	21.58	16	4
145	10.03	5.67	19.62	14.61	16	5
146	27.69	27.9	7.63	5.67	16	6

Table III.1

148	18.31	10.25	*	*	17	1
149	12.64	11.55	21.11	23.33	17	2
151	5.23	6.76	*	*	17	3
152	4.8	5	*	*	17	4
153	4.36	5.45	*	*	17	5
158	*	*	*	*	17	6
159	*	*	*	*	18	1
161	20.11	19.62	*	*	18	2
163	*	*	*	*	18	3
164	2.4	2.18	2.62	2.62	18	4
165	24.42	25.94	4.58	6.76	18	5
167	10.9	12.86			18	6
169	9.81	10.46	15.48	14.82	19	1
170	12.21	9.16	10.03	10.25	19	2
173	7.85	8.5	*	*	19	3
176	15.91	18.53	*	*	19	4
178	*	*	*	*	19	5
180	3.05	3.49	*	*	19	6
181	30.52	27.25	25.07	24.85	20	1
189	6.76	7.85	*	*	20	2
191	7.19	13.08	16.13	17.22	20	3
193	5.01	4.58	*	*	20	4
199	20.93	25.07	19.62	22.89	20	5
201	*	*	*	*	20	6
202	9.37	10.68	17.66	24.72	21	1
203	6.32	8.07	10.68	12.43	21	2
204	*	*	*	*	21	3
205	5.23	6.1	27.25	21.8	21	4
207	*	*	*	*	21	5
208	*	*	*	*	21	6
209	4.58	3.49	29.21	28.12	22	1
211	*	*	*	*	22	2
212	11.12	9.37	22.45	21.36	22	3
213	14.17	12.86	*	*	22	4
214	*	*	*	*	22	5
215	28.78	28.78	19.84	19.62	22	6
217	10.46	8.5	6.32	6.76	23	1
218	28.12	25.51	*	*	23	2
224	1.74	1.74	18.53	18.75	23	3
227	*	*	*	*	23	4
230	6.98	9.16	*	*	23	5
231	11.55	11.55	*	*	23	6
236	*	*	*	*	24	1
238	*	*	*	*	24	2
239	30.3	30.52	35.75	36.62	24	3
240	*	*	*	*	24	4
242	*	*	*	*	24	5
243	15.48	23.33	5.45	5.23	24	6
247	17.66	22.02	22.02	21.8	25	1
248	10.68	21.36	*	*	25	2
251	1.31	1.31	*	*	25	3
253	*	*	*	*	25	4
255	2.83	2.62	7.19	5.89	25	5

Table III.1 cont.

256	30.96	30.96	*	*	25	6
257	23.76	23.98	*	*	26	1
258	*	*	*	*	26	2
259	*	*	*	*	26	3
261	*	*	*	*	26	4
263	10.9	9.16	*	*	26	5
264		15.04	16.57	17.22	26	6
265	29.43	29.21	*	*	27	1
266	*	*	*	*	27	2
269	11.37	11.99	14.61	16.35	27	3
270	11.12	11.12	27.69	22.89	27	4
272	17.22	19.62	14.61	14.17	27	5
273	33.57	35.97	*	*	27	6
277	14.17	20.27	39.46		27	10
279	10.25	9.37	*	*	27	9
281	12.86	11.99	10.68	10.25	27	8
282	*	*	*	*	27	7
285	*	*	*	*	26	10
286	10.25	13.08	28.56	23.98	26	9
287	29.43	31.61	*	*	26	8
288	28.34	26.16	13.08	14.61	26	7
289	22.67	25.94	*	*	25	10
290	8.28	13.52	4.58	5.45	25	9
291	*	*	*	*	25	8
292	*	*	*	*	25	7
293	25.51	22.45	11.34	11.55	24	10
297	*	*	*	*	24	9
298	4.36	5.45	*	*	24	8
300	*	*	*	*	24	7
302	*	*	*	*	23	10
303	2.18	2.4	*	*	23	9
306	7.14	8.5	*	*	23	8
307	39.68	37.93	*	*	23	7
308	*	*	*	*	22	10
309	3.92	4.36	*	*	22	9
311	16.79	16.57	6.76	4.88	22	8
312	3.05	3.27	*	*	22	7
313	6.32	5.67	*	*	21	10
316	13.95	14.82	11.34	7.85	21	9
317	7.41	5.23	*	*	21	8
319	3.05	3.05	4.36	4.58	21	7
322	8.72	9.37	4.36	3.71	21	10
323	3.49	2.83	7.19	9.81	20	9
324	9.81	9.59	*	*	20	8
325	10.46	11.77	*	*	20	7
326	3.05	3.05	1.96	2.18	19	10
328	*	*	*	*	19	9
330	9.59	8.07	14.17	13.95	19	8
331	11.99	13.08	*	*	19	7
332	4.36	3.92	7.19	7.19	18	10
333	6.32	6.54	7.63	8.5	18	9
334	9.81	10.03	11.55	10.46	18	8
335	8.72	5.89	10.25	9.81	18	7

Table III.1 cont.

336	11.34	9.37	12.64	13.52	17	10
337	16.35	17.22	24.2	26.38	17	9
342	13.52	14.82	*	*	17	8
343	8.07	7.63	*	*	17	7
344	*	*	*	*	16	10
345	*	*	*	*	16	9
346	7.19	7.41	12.43	14.82	16	8
347	*	*	*	*	16	7
350	*	*	*	*	15	10
351	*	*	*	*	15	9
352	3.49	3.49	9.16	9.59	15	8
354	*	*	*	*	15	7
355	9.16	11.12		7.85	14	10
357	7.19	6.76			14	9
360	12.21	12.64	12.86	12.43	14	8
364	*	*	*	*	14	7
365	11.34	10.03	*	*	13	10
368	11.34	10.9	*	*	13	9
369	14.39	15.48	26.81	21.58	13	8
370	19.4	18.5	8.5	8.07	13	7
372	*	*	*	*	12	10
375	34.88	24.85	26.16	25.72	12	9
377	28.99	29.65	*	*	12	8
378	25.72	31.61	39.02	21.76	12	7
380	32.7	32.7	29.43	30.3	11	10
381	3.27	4.36	3.27	7.19	11	9
383	36.19	27.47	32.26	36.19	11	8
384	5.45	5.01	18.97	20.93	11	7
385	13.73	33.14	39.89	35.32	10	10
386	9.37	4.8	28.78	24.63	10	9
388	32.92	32.7	36.6	30.3	10	8
391	*	*	*	*	10	7
393	17.44	7.19	*	*	9	10
394	33.35	37.06	38.88		9	9
395	3.27	4.8	*	*	9	8
397	16.79	18.31	*	37.93	9	7
399	34.23	26.81	33.79	31.61	8	10
401	9.81	10.9	24.2	32.7	8	9
403	4.58	5.23	*	*	8	8
404	36.41	34.66	23.54	20.06	8	7
405	35.32	34.01	11.99	11.55	7	10
406	24.85	26.16	*	*	7	9
407	39.68	34.88	*	*	7	8
408	11.77	13.7	15.02	12.86	7	7
409	25.29	27.25	*	*	6	10
411	25.51	17	39.46	39.46	6	9
412	45.78	30.46	8.72	5.89	6	8
413	8.07	7.63	*	*	6	7
414	*	*	*	*	5	10
415	*	*	*	*	5	9
417	17.44	29.65	20.49	18.31	5	8
422	25.94	26.16	23.76	20.06	5	7
423	*	*	*	*	4	10

Table III.1 cont.

424	*	*	*	*	4	9
425	3.71	6.76	*	*	4	8
426	7.41	6.54	16.57	16.13	4	7
427	14.39	12.64	*	*	3	10
429	*	38.59	*	*	3	9
430	*	*	*	*	3	8
431	*	*	*	*	3	7
434	8.5	6.76	8.07	7.63	2	10
438	25.29	19.84			2	9
443	17.66	28.12	17.22	13.3	2	8
445	10.9	13.73	27.03	34.88	2	7
446	15.48	19.4	*	*	1	10
447	15.91	12.21	21.58	19.4	1	9
448	11.55	6.32	15.26	13.3	1	8

APPENDIX IV

Table IV.1 Size of ripe fruit from the F_1 generation. Asterix indicates that no is data available.

	Fruit 1			Fruit 2		
Plant Number	Width (mm)	Width (mm)	Height (mm)	Width (mm)	Width (mm)	Height (mm)
1	23	23	34	23	23	29
3	41	22	48	26	24	35
5	22	21	35	22	20	31
6	31	29	34	31	29	36
7	25	25	31	25	25	32
8	36	21	37	*	*	*
10	35	31	46	24	22	32
12	31	27	32	21	20	25
13	36	37	51	*	*	*
14	27	25	30	*	*	*
16	23	22	32	21	20	27
17	26	25	40	27	30	46
19	24	21	34	17	17	31
21	30	27	35	28	27	35
24	31	25	38	29	26	37
25	27	27	26	28	27	35
28	29	23	35	24	21	33
29	21	21	29	17	22	30
30	27	25	34	25	23	33
31	36	36	50	*	*	*
32	19	18	30	17	15	28
33	41	36	44	30	28	35
34	35	35	49	20	21	33
35	29	28	34	*	*	*
36	27	26	37	24	23	30
37	28	26	41	30	30	33
42	19	18	30	19	18	32
43	34	31	42	23	23	31
44	31	25	28	20	18	28
45	21	25	25	*	*	*
46	43	31	46	31	28	41
47	32	27	32	22	22	29
48	23	21	31	25	25	40
50	25	25	32	*	*	*
52	24	24	29	23	24	31
53	23	22	28	23	21	28
54	23	23	33	23	22	33
55	34	31	46	29	25	33
56	31	31	23	26	25	29
57	15	13	17	*	*	*
59	26	23	35	26	26	33

Table IV.1 cont.

60	24	22	30	22	22	25
61	24	22	34	20	18	24
62	26	25	27	20	20	28
64	33	38	40	23	22	29
65	32	28	41	25	25	34
70	18	18	28	28	25	37
71	26	25	28	*	*	*
72	30	28	30	28	26	32
74	21	22	29	25	25	33
75	40	27	49	28	25	43
76	36	34	43	25	23	29
82	25	26	36	28	35	39
83	39	26	47	23	22	33
84	27	24	37	25	23	36
85	30	27	35	*	*	*
86	20	20	27	22	15	26
87	28	27	32	27	28	24
92	25	25	29	26	24	29
94	29	28	36	28	28	38
97	33	38	46	25	23	29
99	25	24	29	25	23	29
100	26	25	40	26	26	41
102	39	26	42	*	*	*
103	21	20	24	*	*	*
109	30	29	40	27	27	32
111	48	29	45	33	25	42
112	33	30	38	26	26	33
113	27	28	36	27	24	36
114	26	28	30	21	20	23
117	26	24	37	*	*	*
118	22	24	46	21	22	35
119	27	22	32	26	25	33
120	23	23	30	23	23	26
121	17	17	24	20	20	31
122	45	36	55	24	22	30
123	26	23	32	27	23	27
124	28	25	43	27	26	41
125	30	26	34	26	25	30
126	22	23	33	25	25	35
127	28	25	31	24	25	30
128	27	27	30	31	30	37
129	28	24	36	26	23	35
130	33	30	36	22	21	28
131	33	20	43	*	*	*
132	33	30	34	24	23	30
133	33	25	38	25	25	36
134	32	29	43	27	26	37
135	29	28	45	26	33	42
136	21	19	28	23	22	31
137	23	27	30	23	22	27
138	40	28	48	27	31	37
139	24	26	34	22	23	39
144	23	23	28	24	23	28

Table IV.1 cont.

145	25	20	31		*	*	*
146	22	21	30		21	21	29
148	21	20	25		50	37	50
149	24	24	30		24	21	29
151	17	17	24		22	21	30
152	27	23	37		22	24	27
153	20	19	26		19	20	28
158	36	32	45		23	23	29
159	32	28	37		31	29	42
161	26	27	44		21	20	30
163	24	22	30		*	*	*
164	24	25	26		24	25	27
165	42	31	48		32	30	38
167	37	35	50		29	29	40
169	22	21	28		18	16	25
170	30	28	32		26	26	30
173	29	28	32		*	*	*
176	32	30	34		*	*	*
178	21	20	27		27	22	27
180	25	25	27		*	*	*
181	30	32	32		*	*	*
189	20	20	24		20	19	25
191	23	24	22		26	25	26
193	35	32	42		*	*	*
199	35	26	40		21	19	36
201	24	22	30		25	25	29
202	42	27	42		26	26	34
203	26	25	31		26	24	25
204	21	23	21		32	28	28
205	25	22	31		20	22	28
207	42	23	44		36	31	41
208	21	22	24		20	19	24
209	26	25	32		25	23	29
211	21	22	23		21	22	31
212	29	29	35		22	23	29
213	21	20	35		26	26	34
214	29	26	34		23	24	29
215	25	25	33		*	*	*
217	22	22	30		20	20	27
218	33	27	41		30	26	36
224	26	27	37		36	33	45
227	27	24	35		29	28	27
230	27	27	38		25	24	29
231	31	28	36		*	*	*
236	29	26	28		*	*	*
238	22	21	29		*	*	*
239	27	26	35		26	23	31
240	23	21	30		22	23	35
242	29	28	35		27	26	29
243	29	25	33		23	24	28
247	43	32	45		28	25	27
248	23	22	29		22	23	26
251	35	28	39		26	24	31

Table IV.1 cont.

253	28	30	45		*	*	*
255	37	26	37		30	28	26
256	32	19	36		30	28	35
257	38	31	52		27	27	41
258	32	28	40		27	25	35
259	26	22	31		*	*	*
261	28	26	34		25	22	26
263	27	26	34		22	21	24
264	22	21	29		21	20	31
265	30	25	42		26	24	33
266	33	29	41		21	20	25
269	22	20	30		20	19	26
270	35	34	46		*	*	*
272	26	26	44		*	*	*
273	34	34	44		23	23	24
277	23	23	38		25	27	29
279	23	21	28		22	21	23
281	23	22	30		22	20	28
282	30	26	31		25	24	28
285	23	22	35		*	*	*
286	27	29	37		*	*	*
287	19	18	24		21	20	27
288	23	20	38		27	26	44
289	25	30	46		20	18	29
290	37	35	48		*	*	*
291	21	19	25		20	19	26
292	23	19	32		25	21	32
293	26	26	35		23	21	33
297	19	21	37		21	21	36
298	33	32	39		23	23	30
300	23	21	28		*	*	*
302	28	26	37		22	20	33
303	25	23	30		26	24	30
306	25	24	34		22	20	29
307	29	28	42		25	26	35
308	32	28	46		26	25	28
309	34	32	36		32	30	36
311	21	18	25		22	20	25
312	20	20	26		*	*	*
313	25	25	33		23	21	27
316	25	23	31		22	21	20
317	24	24	26		23	23	24
319	25	21	30		25	24	31
322	28	22	39		27	28	40
323	32	15	39		*	*	*
324	32	30	43		25	26	36
325	26	25	34		*	*	*
326	25	22	27		20	20	27
328	25	24	31		*	*	*
330	31	32	41		24	26	35
331	31	29	43		27	26	42
332	23	21	28		22	22	29
333	30	27	35		23	24	30

Table IV.1 cont.

334	26	25	30	27	22	33
335	34	22	43	26	25	35
336	20	20	22	19	19	19
337	30	26	34	27	26	36
342	37	37	44	28	25	32
343	30	26	43	*	*	*
344	20	19	24	*	*	*
345	24	23	36	24	23	34
346	27	24	38	27	27	38
347	30	27	35	25	26	32
350	25	24	33	21	20	30
351	27	27	31	25	26	28
352	29	27	35	25	22	28
354	26	25	31	*	*	*
355	26	25	29	24	22	26
357	24	24	29	*	*	*
360	26	28	30	*	*	*
364	26	28	36	22	22	35
365	21	29	40	23	21	30
368	33	32	50	21	21	41
369	28	28	41	27	28	31
370	27	27	52	22	24	49
372	30	31	39	28	27	36
375	25	24	43	22	23	38
377	22	24	39	26	27	34
378	30	31	40	30	32	47
380	30	27	29	28	27	27
381	25	21	31	29	23	32
383	30	28	29	25	25	27
384	29	28	36	*	*	*
385	26	25	42	25	25	40
386	31	20	27	21	20	22
388	24	25	36	27	27	27
391	25	25	36	24	21	33
393	18	18	34	*	*	*
394	22	22	29	22	20	31
395	34	30	41	20	20	29
397	25	22	32	30	28	45
399	27	27	36	27	25	34
401	32	28	40	28	23	31
403	29	28	36	31	36	35
404	28	25	37	29	25	35
405	20	18	35	27	22	29
406	27	27	36	21	19	37
407	27	27	36	30	30	40
408	37	32	33	28	29	27
409	33	27	32	26	26	26
411	23	21	23	24	23	28
412	30	31	44	28	27	37
413	28	27	36	25	23	35
414	26	25	36	27	23	38
415	43	32	38	29	28	29
417	20	18	20	22	22	30

Table IV.1 cont.

422	39	31	34	*	*	*
423	24	25	33	23	21	31
424	32	30	47	21	20	29
425	25	25	32	26	25	31
426	23	24	36	20	21	29
427	30	27	48	27	28	38
429	20	21	28	25	24	29
430	24	24	34	22	23	26
431	23	21	36	23	23	25
434	26	25	30	29	25	35
438	15	15	22	31	34	39
443	29	27	37	21	20	27
445	25	23	34	23	23	30
446	20	20	29	*	*	*
447	25	20	23	20	20	25
448	32	30	40	27	26	32

APPENDIX V

Table V.1 Size of unripe fruit from the F_1 generation. Asterix indicates that no is data available.

	Fruit 1			Fruit 2		
Plant Number	Width (mm)	Width (mm)	Height (mm)	Width (mm)	Width (mm)	Height (mm)
1	20	20	27	16	18	23
3	24	24	33	16	16	24
5	19	21	31	24	21	38
6	19	18	22	17	17	22
7	18	18	23	19	18	26
8	*	*	*	*	*	*
10	20	18	24	19	18	25
12	25	21	25	18	18	23
13	20	18	28	22	21	40
14	*	*	*	*	*	*
16	*	*	*	*	*	*
17	22	21	27	21	18	26
19	18	17	28	15	14	21
21	17	17	25	15	14	21
24	16	15	19	*	*	*
25	20	18	18	24	21	38
28	16	14	21	19	17	24
29	14	14	18	14	13	17
30	18	17	26	15	14	23
31	20	19	30	14	13	17
32	16	17	20	15	15	19
33	18	19	23	17	17	21
34	15	14	24	14	14	24
35	*	*	*	*	*	*
36	17	16	26	17	15	26
37	14	13	19	15	12	15
42	*	*	*	*	*	*
43	22	21	30	22	21	27
44	20	18	27	*	*	*
45	17	16	18	17	14	25
46	17	17	25	17	15	23
47	20	23	33	17	14	22
48	23	21	34	17	18	31
50	*	*	*	*	*	*
52	18	17	24	14	15	24
53	23	22	33	18	17	24
54	18	16	29	18	15	28
55	21	19	32	17	16	29
56	20	21	21	17	18	19
57	18	17	22	14	16	18
59	18	18	25	20	20	30
60	20	23	26	*	*	*
61	*	*	*	*	*	*
62	16	21	25	18	17	20
64	23	22	33	20	22	29

Table V.1 cont.

65	*	*	*		*	*	*
70	20	18	27		20	17	26
71	19	17	22		19	15	21
72	23	24	30		20	22	26
74	20	19	25		17	18	24
75	17	14	24		15	15	24
76	20	17	26		18	18	24
82	16	19	31		24	21	36
83	*	*	*		*	*	*
84	20	18	29		17	17	23
85	26	23	34		19	19	26
86	15	15	22		*	*	*
87	17	16	18		15	13	16
92	20	18	22		19	18	21
94	20	20	29		21	19	25
97	17	16	21		*	*	*
99	18	18	26		*	*	*
100	21	20	30		20	19	30
102	*	*	*		*	*	*
103	*	*	*		*	*	*
109	19	18	29		18	18	26
111	21	20	29		18	19	26
112	22	23	31		21	19	26
113	22	20	22		17	16	24
114	20	19	26		22	18	26
117	*	*	*		*	*	*
118	*	*	*		*	*	*
119	*	*	*		14	13	16
120	*	*	*		*	*	*
121	*	*	*		*	*	*
122	*	*	*		*	*	*
123	15	15	20		15	13	18
124	18	15	26		17	16	25
125	20	17	19		*	*	*
126	*	*	*		*	*	*
127	18	17	22		15	15	28
128	15	16	24		17	15	23
129	18	18	24		*	*	*
130	*	*	*		*	*	*
131	17	17	27		19	18	26
132	21	20	27		20	19	27
133	17	16	21		15	14	18
134	19	19	27		14	14	18
135	21	22	33		21	20	30
136	20	21	30		18	15	22
137	14	13	15		*	*	*
138	20	19	26		*	*	*
139	14	13	19		15	14	20
144	19	17	24		15	15	22
145	21	18	23		17	15	24
146	18	17	28		19	19	27
148	21	17	21		*	*	*
149	23	21	21		20	18	21

Table V.1 cont.

151	15	14	22		*	*	*
152	18	17	25		*	*	*
153	18	17	25		*	*	*
158	*	*	*		*	*	*
159	*	*	*		*	*	*
161	15	15	21		*	*	*
163	*	*	*		*	*	*
164	18	20	24		17	20	21
165	17	16	24		26	26	36
167	23	22	33		*	*	*
169	15	15	23		15	13	20
170	24	23	30		22	21	27
173	17	17	20		*	*	*
176	20	20	26		*	*	*
178	*	*	*		*	*	*
180	22	20	24		*	*	*
181	20	20	22		21	20	22
189	15	14	14		*	*	*
191	25	25	29		20	19	23
193	22	22	28		*	*	*
199	15	15	26		15	15	25
201	*	*	*		*	*	*
202	17	15	21		18	17	24
203	17	17	21		17	16	21
204	*	*	*		*	*	*
205	17	18	24		14	14	20
207	*	*	*		*	*	*
208	*	*	*		*	*	*
209	26	25	27		18	15	26
211	*	*	*		*	*	*
212	16	15	19		14	13	19
213	17	15	30		*	*	*
214	*	*	*		*	*	*
215	18	18	26		15	15	22
217	18	17	26		17	14	23
218	18	16	20		*	*	*
224	23	20	31		15	15	20
227	*	*	*		*	*	*
230	20	19	18		*	*	*
231	18	17	15		*	*	*
236	*	*	*		*	*	*
238	*	*	*		*	*	*
239	19	15	24		16	15	20
240	*	*	*		*	*	*
242	*	*	*		*	*	*
243	19	17	24		22	19	24
247	22	20	27		20	16	29
248	17	17	20		*	*	*
251	22	22	26		*	*	*
253	*	*	*		*	*	*
255	22	21	24		20	19	23
256	19	16	23		*	*	*
257	18	18	21		*	*	*

Table V.1 cont.

258	*	*	*		*	*	*
259	*	*	*		*	*	*
261	*	*	*		*	*	*
263	19	17	19		*	*	*
264	15	14	23		22	19	25
265	14	13	15		*	*	*
266	*	*	*		*	*	*
269	18	16	30		18	15	28
270	20	18	23		15	13	17
272	18	15	23		19	16	28
273	14	14	18		*	*	*
277	15	16	21		13	13	15
279	18	17	24		*	*	*
281	16	15	24		15	14	23
282	*	*	*		*	*	*
285	*	*	*		*	*	*
286	19	19	28		15	15	21
287	13	12	20		*	*	*
288	17	15	26		15	15	25
289	14	14	21		*	*	*
290	23	23	36		17	16	22
291	*	*	*		*	*	*
292	*	*	*		*	*	*
293	14	14	26		15	14	25
297	*	*	*		*	*	*
298	19	17	21		*	*	*
300	*	*	*		*	*	*
302	*	*	*		*	*	*
303	23	20	25		*	*	*
306	21	22	26		*	*	*
307	17	13	14		*	*	*
308	*	*	*		*	*	*
309	16	15	17		*	*	*
311	15	12	21		16	13	24
312	16	17	22		*	*	*
313	14	14	18		*	*	*
316	20	21	23		17	16	19
317	15	16	22		*	*	*
319	18	17	22		15	17	23
322	16	19	34		17	15	27
323	15	15	27		13	13	24
324	15	13	19		*	*	*
325	15	15	21		*	*	*
326	20	19	25		17	17	23
328	*	*	*		*	*	*
330	15	17	25		15	15	21
331	20	19	28		*	*	*
332	16	16	23		15	13	20
333	20	18	29		17	17	25
334	18	17	24		17	15	22
335	15	12	22		15	17	25
336	18	19	28		19	14	22
337	25	24	29		20	20	25

Table V.1 cont.

342	14	14	19		*	*	*
343	20	20	31		*	*	*
344	*	*	*		*	*	*
345	*	*	*		*	*	*
346	19	18	26		17	16	26
347	*	*	*		*	*	*
350	*	*	*		*	*	*
351	18	15	22		15	15	21
352	*	*	*		*	*	*
354	*	*	*		*	*	*
355	21	18	32		19	22	30
357	18	18	23		*	*	*
360	15	15	25		14	15	20
364	*	*	*		*	*	*
365	17	16	22		*	*	*
368	16	16	29		*	*	*
369	20	21	21		17	17	26
370	17	17	31		16	16	28
372	*	*	*		*	*	*
375	15	15	25		16	15	25
377	14	14	19		*	*	*
378	20	19	32		17	16	26
380	18	17	21		17	19	19
381	19	18	28		16	17	26
383	18	18	25		18	16	23
384	18	18	25		15	14	24
385	15	15	27		17	15	26
386	17	16	26		16	13	20
388	17	18	26		17	16	20
391	*	*	*		*	*	*
393	15	15	20		*	*	*
394	16	15	21		13	13	20
395	19	18	19		*	*	*
397	22	20	29		15	15	25
399	17	16	22		16	14	22
401	15	15	24		15	14	19
403	20	18	24		*	*	*
404	19	16	28		20	19	34
405	15	15	18		22	19	24
406	16	14	27		*	*	*
407	16	15	23		*	*	*
408	17	15	17		20	14	17
409	18	17	19		*	*	*
411	18	17	30		17	15	26
412	15	15	23		15	14	22
413	17	16	26		*	*	*
414	*	*	*		*	*	*
415	*	*	*		*	*	*
417	17	15	21		18	18	25
422	17	17	23		18	16	22
423	*	*	*		*	*	*
424	*	*	*		*	*	*
425	17	17	22		*	*	*

Table V.1 cont.

426	17	16	25		15	13	19
427	21	19	30		*	*	*
429	17	16	26		*	*	*
430	*	*	*		*	*	*
431	*	*	*		*	*	*
434	21	20	24		17	17	21
438	19	17	23		*	*	*
443	20	20	24		19	19	25
445	22	21	33		19	19	24
446	15	15	19		*	*	*
447	19	15	24		15	15	20
448	18	17	26		16	16	21

APPENDIX VI: Sequences of clones identified from the suppressive subtractive hybridisation.

Clone Sequnce

FFC18

NCCCTCGACGCTCCCGCTCCACNAATTNTGGGGCCNCCNGGGGCTNNCTCAACCCGGNTANAANCNTNNNCGNCNTCNNC
NCGACCTCTGGNCNTCCCCNAATNNCCCNGCNGGANNCTNCGANCGCCGTCACTNCANCCNCNNNGGNTTCACNCATNGG
CCTNCNNCTNACCCGNTCAACGGCCGNGGANGCTGCCCCCAACCCGGCTANNNAAATCCTTACNNCNTCGCTCAAATNGG
GGTTNCTTTNNCGGGAAAANNGGCNNCNACCTTAACNCAATTNNGCGAGTNNNCNGNCAAATCNNAAAACTTTNCCCNGN
ANCCCGNGNNNNGTANTTNCNTTAAANCTGGCGAANTAATCCNGGGGGTCCAANGNNGATCCNGNNATCCCNNCCGTTCC
TCCCCCTATNTCGGGGGCCTNTCCCTANTTTNGGTCCTCGGNGGATACCCGNNCGCCGGCGGTTTCCGGGGCTTATNNNG
NGCGGCCNGTCCAGGGGAGTCACTTTCCCCGGAAATCCTGGTAAACCTAACTACGGTANACAAANGTTTCCTAAACTTTC
NTNTCNGTCGTNAGAAGNATATCTTAAGAAGTTCAGAGNGTCGNTATTGTNGCTTATACCCAAGAATACACGTCACNGGN
TTCGTCCAGAAGGGATTTGGGGTCTATTACTATACNGTNGAATACCTTGNAAACATTTGCACCTAGGGGAAGTACTTNTT
AACAGTNATTCTTCGAACCAACTAGTGAGATATCACCTCTCAAAACCACCTTTTCCGTTCCGCACGTATNGTCNATCCCA
CATAGGATGTAACCGACAACTACCACTATGGGTAAATATACGAAAGTTAATACCTTCACTCTCACAGTTTTAGCGTCCTT
CACGTACCTCGTACTCTNGACGATTTTAGTTAACTAGTTCNAATCTTTTCTTCACCCCTCCAACATAACTAAGAACATAC
ACTGAGTAGATGAATATCACGGAGAATTTAANCTATTAACTATATTCTCAACTTATTTGTTCACANTCGGTTAAAAAACA
CGNACGTAGTTTTTTTTTNN

FFC20

ACCATGACACAAGCTTGACAAAGTTGTCGTTCAATGCAATTCCAGCCTTGGCATCAAAGATGCTTGACCTGTTGTCACCA
ATGAAGTCGGTTGACACAACATCATCTTCGGTGTAACCCAAGATACCCTTCATCTTTCCCTCAGACTCCTCCTTGATAGC
AGCCTTGATCTGGTCATAGGTGGCCTTCTTCTCAAGTCTGACAGTGAGGTCAACAACTGAAACATCAACAGTGGGTACCG
CCCTCCGCACCACGCCCTGGGCAGGACCATGACACAAGCTTGACAAAGTTGTCGTTCAATGCAATTCCGGCCTCGGCATC
AAAGATGCTTGACCTGTTGTCACCAATGAAGTCGGTTGACACAACATCATCTTCGGTGTAACCCAAGATACCCTTCATCT
TTNCCTCAGACTCCTCCTTGATAGCAGCTTGATCTGGGCATAAGTGGCCTTCTTTTCAAGTCTGACAGTGAGGTCAACAA
CTGAAACATAACAGTGGGTACCGGCCTTCGGAACCANGCCCTGGGCTAAACGGGCGGCAACGCGGTGGAACTTCAGCTTT
GGTCCTTTAATGAGGGTNAATGCCCTTGGCGGNATATGGGCATACTGGTTCTGNGGGAAATGTATTCGGTTACAAATTCN
CACAACATACNAANCCGGAACCTAAAGNGTAAAGCCTGGGGGCCTAATGAGNGAGCTTAATTACATTTAATGNGTTGGCT
AATTGCCCGTTTCAATCNGGGAAAACTGCGNGCCAANTGCATTAATGAATTGGCCANNCCCCGGGGAAAAAGCGGTTGCG
ATTGGGCGCTTTTTTCGGTTCCTGGTTANNTGNTNNNTTGGNCTTNGGCGTCGGTTTGGGNAAGNGGTTAANTTNCTTNA
AGGNGGGATNCGGTTTCCCCAANCNGGGGTAACCCCGGAAAAACATTGGCCAAAGGCCCNAAANGGCCCGAACCTNNAAA
AGGCCCNNNNCTGGGTTTCNAAGTTCCCCCTGGGAGGTTCAAAAATNCCTTCNNTNAAGGGGAACCCCGGTTTTANAAAC
NGGTTNCCCGNN

FFC22

GGAACAGTGAAGTTGTCCGTATGAGTATCCAAGAAATGTCCATCAAAGGTGATGAACTACCACCTGAATATATTGNCACA
AAAAGCAAGGTTGGAGCCATANANANCTNACCAGAATTGGGGCAAAATCCCACCATTGATGTTAGTCTCTTCTCCAATCA
TCAATTGGCACTAAGGAAGCAGAGATCGAACTAAAGAAACTCAGATCAGCTCTTAGCTCATCCGCCTGCTTCCAGGCAAT
CGGTCATGGGATTTCAAGTTCCTTTCTGGACAAGGTACC

FFC41

ACCATTGCAAGGTCAGGAACCAAGGAATTCATGGAAGCCTTGGCTGCTGGTGCTGACGTTAGCATGATTGGTCAGTTTGG
TGTTGGTTTCTACTCTGCCTATCTTGTTGCTGAGAAGGTCATTGTCACCTCCAAGCACAATGATGACGAAGCAGTACCTG
CCCGGGCGGNCGCTCGAGGGCTANAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATT
GCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTTCACACAACATACNANC
CGGAAGCATAAAGTGCAAAGCCTGGGGTGCCTAATGAANTGAGCTAACNCACATTAANTTGCGTTTGNGCTCACTGCCCG
CTTTCCANTNAGGGAAACCTGNTCGTGCCCAACCTGCATTAATGGAATCGGTCAANGCNCGGNNNAGAAGGCCCGTTAGT
GTAATTNGGTGCTTCTTCGGTTTTCTTTNCTCAATTGACTCCGCTTGCTCCTCTGNNNTNTTGGCTTGNTGNCNAANTGN
TANTCANCTCACTTNANNAGTCGGTAANTACCNNNNTTNCANNTAAATNNGGGGNATANCGCTAGGAAATCAAACCATNT
GNTANCACNAAANGCCCNNCAAANNGCCANNAANCCGNANAAAAAANGNTCTANTTTNGTTNNCCTTTTTTCCNTAANGCT
TTNNCCTCCTTGAACNANNNNTCTNNGANATNCNTCTNANTNCTNTANATGCNNTNAACCCGNACNGGNCTTTANGNNTN
CNANCGCTTCTCCCTTGGAACCTTCCTTCCCNGNTTNTCNNTNCCGNCNCTTNGCTTNACCGNNCACAGTCTCCTNTTTT
CCCNTTCNNAATCNCNGTCCNTCTANTTNNCTCTTCCTTNCGNNTTNTCCTTCGCCGTCTGCTCGTTAGCTNTCANTNGC
CTTTNGTGTCCATNACNCTCCTNACNCCNACNNCTGATCTTTATAGCNGATNCTANCNCAGTNTCNNNTCCCNTNCAATT
NCTNNTTTCTTNCCCTCTATNTCCTNNNCAGTNTTCTCACCATNTCTACATNCTNTCGTTNCATCNCANNCATCNGTGCT
NACTNTGNNTNTGNTTNNNANTNTCTGATNNCTCTCTCTCCG

FFC47

NCCCCTTTNNTACGATCTTGGNCGTTTAANGNCNTNTTGGGCACGGNNCCAGGTCGACCGTATCTATTNTCTTGATATCT
TANNCCCTTGCNTTCCGGGGGTATCGGCCCACNGNGTGGTGCGNACGGCNGTACTCNCCCTCTTGTCTNTNTCTCGTCCC
ATTGANGATATTCNTCATCCACCTTCTTGATGCTCCCNTTGTTNTTCACNAATCNCCTCCTTNTTCNTCTCTGTNCCTCT
GAANCCCANCTCCTGTAACCCTCCTACTAATATGATNCNNCTCACAATNTTCTCANANATCTTCCTCNAATTGTTCNATT
TTTCTTCTNCCCGGGCGGCCTTTTTNNTGGCTNTATCTCCCTCACCNTACNT

FFC65

ACCACTCAACCAAGAGCGGTGATGAGATGACTAGCCTTAAGGACTACGTCACAAGGATGAAGGAGGGCCAGCAAGACATC
TACTACATCACTGGTGAAAGCAAGAAGGCTGTTGAGAACTCTCCTTTCCTTGAGAAGCTTAAGAAGAAGGGATATGANGT
TCTGTTCATGGTTGATGCTATTGATGAGTATGCAGTTGGTCAGCTCAAGGAGTATGAGGGCAAAAAGCTTGTCTCTGCCA
CCAAGGAAGGTCTCAAGCTTGATGAGACGGAGGATGAGGCAAAGAAGAAGGAAGAGTTGAAGGAGAAGTTTGAGGGACTC
TGCAAGGTTGTCAAGGATGTGTTGGGTGATCGTGTTGAGAAGGTTGTGGNGTCTGACCCGTGTGGGTTGATTCTCCCTGC
TGTTTGGTGACTGGCGAGTATGGCTGGACTGCGAACATGGNANGGATCATGAAAGCCCAGNCNTTTGANGGATAGCACCA
TGGCTGGNTACCATGGTCCTCCCNGAACACCATGGAGNATCCAACCCCGANNAACCCCAATCATGGGAGGAGCTTCAGAA
AGAANGCCTTGTTGCCAGACCANANCCACCAGGTCANTGAAAGGACCCTTGGGCTTCCTTTCTCNTTTGAGACCTGCCCT
TGGTNACCTTCTGGGGTTAATCCTTTGACAACCCCAACAACCTTTGGCACNNGGATCCCACAGGGATGTTGNACCTTCGG
ACTGNGCCTTTGTGGATGAATCCCCTTGANGGCCAACCTTACATGCCNNCACTTGNAGGATGTNNACTCCNNAACCCNTN
ANNNNGATGGNNGNAATTCCTTACCCGNANAATTGNNCNACGNTNAAGNTNNTNTTTGGCTGGGNCCAATTTCTTTTACC
NNTTTATTCNACCTNAANNTNTTTNCTNCCNCNTGATGGCGTCGCCTNCGNCTNCCNNTCNTNTNTTTTCANNCTTTCNC
CCCTCCTNNCTCGNGCTNNGCTACTCGNCNNCNNTCTNCNGCCNNCCCNTCCCTCTTTCCCCNNNNCANCNCTCCCTCNN
CNTCTCCTTCACCNCNCNNCTCCTNCNNCCCCCTCCCNTCNCCCCG

FFC89

GCACTATCGCCTCCTGAGCTGGGTTCTGGAATGTGGCTATGGACAACCTGCTGTGGNTTGAGTTCACCACCGCCTGGTGA
ACAACATTTTTGAACCTTCCATTGNTNAAAAAATGTCATGGGCTTCAAGGNTTACCACGAAAGCTNCTTNCACAGGTTGA
ACGGTGATCCACGTCTTTCGCCGTCCTTGGTGGCCTGAAGTCCACCGACTTGGTCCTGCAGCAAAAGGGTGATGGTACCT
GCGGGGCGGCCGC

FFC95

ACTTCATGGCAGCATCAAAGACGGAAACTTTATCTCCTGTGGGATGATGGATTGTTTTGGGGCCAACTTCTCCTTTCAAA
AACTTATTGACCAGGCGAATATTTGCAAAAGTGCCTCTCGCCATTATTTCATCATTACCACGACGACTACCATAAGAGTT
GAAGTCTCTTCTATCAACACCGCGCTCCATAAGGTATTTGGCTGCTGGACTCTCTTTGTGGATGCTACCAGCTGGTGAGA
TGTGGTCAGTTGTAATGCTATCTCCAAAGTTGAGCAAGCAGTAAGCATTCTTCACACCATGAGGGCCAGGAGGAGACATG
GTCATATCTTTAAAGTAAGGTGGCTCGTGTATATACGTGGACTTTGGGTCCCACACATAAAGATTGCCAGATGGTACCTG
CCCGGGCGGCCGC

FFC96

ACAAGTTTTGCATTTACGAGGAACCAGAAGGAGAATTGTTAGCTCCACATTTTCATGAACATTTTAAATTTATTTAGGGT
TTTATAAATTATTAATGTTCCCCTTTTCTTTAGTTTGAGTGCGTTTCTATTTCTCCTTTATATGAATGGACTCCGACATC
CCAATAATATTGAGTTTCTGTTGTAGAATTTGAGTCACTTTGTATAGGTTGTGTGGAGAGGCTAATAAATTATTTCAATA
CTTCTCTTGTATACCCATTGTTGGGCTGTTTGTTGTTATCATAATAGCAACATGCTGTGTGTGGCCAAATAAACTGTTCT
GCACCTTGTTGGTATGGGAAGACTTTCTGATNATGAANAAAAAAAAAAAAAAAA

FFC104

TTTNAACTCCCTTTTTAATANCCCTTTTNAANCTCCCNNANNATNCNCCCGGNCGCCNNGGCNCGCCGGATTGAATTCCG
GNTGNCCTTTNCGATCTCGGAGTTCANGGGATAGGTNTACCAATTGGGGNNCTTGACATGTATGTAGCANCTGCTGGTAT
CAACCCACAAAGAGTGCTCCCAGTTATGCTTGATGTCGNGACAAACANTCANAANCTTCTANAANATCCTCTTTATCTTG
GACTGCGACAACCTAGGTTNNAAGGNNAAGAATNTTTGNCAATAGTTGATGAATTTGTGGAAGCTGTTCATGCTCGNTGN
CCAAANGCTGNNGTGCATNCNGAGGATTTTCAAGCAAAGNGGNCTTTTGAGACGCTNGATCGCTATCCGGAAAAAGTTNT
GCATGTTCAATGATGATATACAGGGAACAGCTGGTGTTGCTCTTGCTGGGCTAT

FFC105

GAAAGANAGAGAATGTCTGGAGGGATCGCGCGTGGNCGNCTCNCCCCAAGAACCAAAAAAACTGGGNGGGAAGAAANANCC
CCATGGGTTTTTGGGTNANGCCCNAAANCGNTTCAAAAAGGGTTCCGCNAATTTGATGGGGTTGGNTGNGGCNTAAAATC
CCGGGNAAAAGCCTCTACTTGGATTGGGGAAAGGGGGGGAACTATTCACTCACACTCCAGTTCAAGTGAGGATTACCCCA
GCAAACCCCCAAAGTGCAAGTTCCCACAGGGTTTTTTCCACCCTAATGNGNATCCTTCTGGAACCGNTTGCCTTTTCATT
CTCAACGAGGATAAGGGGTGGGAGGGCCCGCCATCACAAGTGAAGCAAATCTTAGTAGGCATTCAGGATTTGCTAGACCA
AGCCTAATGCTGNTGATCCAAGCACAAACTGANGGGGTATCAAGCTCTTTCATCCAGGAACCAGCTGAGTACCTCGGGGG
NCCGGGGCCGNTTCGAAGGGGCTAANAAGCGGGCCGGCACCGGCCGGNGGGAGCTTCCAAGCTTTTTGGTTCCCCTTTNN
TGGAAGGGGNTTAAATTGGCGCCCTTNGGGGGGNAAATCATGGGGCANAAGGCTGGTTTCCTGNGGGGGAAAAATTGNTA
ANTNCCGNTNTANAAATTNCACCACCAACAATACGANCCCGGGGAANCATAAAAAGGGGGNAAAACCTNGGGGGGGGGCC
TAATNGAGGGGGGGGCNAACCTCCNATTAANTGGGGTNGGGCGTCNCTGNCCCCCCTTTTTCANANGGGGAAAACANNNN
GGGGCCCNCTGCGTTTAAANNAAAAAGNCCNCACCCNNGGANGAGNAGGGGNTTTCGNAANNNGGGGGNNTCTTTTTCCC
ATTTCNTTAATAAAAAAACNCAGCCCTNGGNNNCTNTNGGNGNGGNGAGGNGNGGNATTCNTCTNCTTANNGGGGGNAAN
NGNGGTTTNCCAAAAANGGGGGGAANCT

SFC3-3.S4

ACTACGACGGAAGCACCACCTATGGCGGTTACTCTGACATTATGGTGGCCGACGAACACTTCATAGTACTGGACATAAGC
TGCCCATCACACTCTTTTTTCACAATCACTTTATTAAAGAGAAATCAACAAAGATGCTCCGAAAACATTTGCAGCAGTCT
GTCAACAATAAATCCCTTGATGATTTCCACTCTTGGTTCTTCAACAAGAGATCTAATGATGGTCGTCATGCCCATGACCA
TGATCATCATGGCCCTCCCAGGGGTGTCTCCAACCCAGCACTACAGGACCATCTTGCTTGGCTCTGTAAAGTACCTGCCC
AGGG

SFC3-3.1

AGCGTCCACNACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGA
ATTCCTGCAGCCCGGGGGATCCGCCCAGGGCGTGGTGCGGAGGGCGGTACCCGAAGTCCCGCTTCTGCCGCGGAGTCCCC
GACCCGAAGATCCGAATCTACGACGTCGGCATGAAGAAGAAAGGCGTCGACGAGTTCCCGTTCTGCGTCCACCTCGTCTC
CTGGGAGAAGGAGAACGTCTCCAGCGAGGCTCTTGAGGCGGCGCGTATCGCGTGTAACAAGTACCTGCCCGGGCGGCCGC
TGGAGGGCTACAGCGAGCCGCCACCNCGGNGGAGCTACATNCTTTTGTTNNCTTNATTTGAGGGNNTAATTGNCGCTTGC
TTGTANATNATGGTCATANATNGCTGCCTGCTGANNNATGTTATCNNN

SFC3-3.52

ACCCGAAGTCCCGCTTCTGCCGCGGAGTCCCCGACCCGAAGATCCGAATCTACGACGTCGGCATGAANAAAAAAGGGGTC
GACCAATTCCCCGTCTGCGTNCACCTCGTCTCTGGGAGAAGGAGAACGTCTTCAGCGAGGCTCTTGAGGCGGCGCGTATC
GCGTGTAACAAGTACCTGCCGGGGCGGCCGCTCGA

SFC2-48

TTNAANNCCCNTTTTTGACGTCCANTACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCG
ATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCGCCCAGGGCGTGGTGCGGAGGGCGGTACCCAAAAATCTTGAA
GAGGAAACATGGGAAATACTGAAGTCTGCCATTTGTGCTATATTCGAAAAGANACCCGTTTCATGCGTNTTGGAGGAACT
NTATCAGGCTGTTAATAATCTTTGNGNGCACANNATGGGAGGGAGTCTGTATCAGCGANTCGAAANAGAGTGCGAAANAC
ACNTATGCAGAGGCACTGCATCATTGNTTGGCCAAAGCCCACATTTGGTGGATGCTCNTATTACTGGTCCCTTATATGGA
TGGTTAGATCGTCTGCTCTCACGTATTCATGGATCATACGCTGTATCCTCTGTAANNTGGTATATC

SFC3-3.88

CGGCTCTTCCTATCATTGTGAAGCAGAATTCACCAAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGCTT
AGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGACAGTGTCGCAATAGTAATTCAACCTAGTACCTGCCC

SFC3-3.S12

ACAATCCTCCTCTTTCTTATTCTTCTTTCTCAGTCGTCACTTTCACCTTAATTTCACCTAGTAGTAGTAGTAGTTACCAT
GCATATACATTCCTTAATCTTTCTCGATCAGCAAGCGTANAACTCGACGAGCTCGTTCATCNATTCCGGCTGAGGATCGG
CGTTCTCGAGCATCCAGANCAGGTGCTCGAACAACACCACCTCGCATGCGACNTTNATTGAGTCGTCGTCGTCGGATCTC
TCTGCCAGCTCCTTGAAAAGGGGGTGGTGGACNACGTCGGGGCCNACGANGTACCGTCTTCTATACTTGCCGACNTATAC
AGCCTGAANGTCTCCAGATGANACTACCTCTTTGGANGATGANTNCTGACTTCTTCNTCCGCCTGANGCTANGGACANAT
CTGTNGCGGTTGCNTTTGCTGAATTAATTCCACTTCTTGCAGGACTGACTTGACCTTCNTTAGCTTTCCCACCTTTCGCC
CTCTTGATNAAACATNATGAATTTTTTAGCAANCNTATTTTTTCNNACAAAACATAATANTTTTGATGNAAGGTTGA

SFC3-S15

ACAGCTACAAGTGACACTCGTGAACTGCGAACTCCTCAAGGCGTCGGCGCGTGAACTGAGAGCCTTATCAGAAAAGCCTA
GAGGTTTGGAATCTTTGTCGTCCGGCAGCGGAGTGAAGTGGCGAACACGGAGACGGCAGAGAAGAGCTGGGAGACTGGAG
AGGATGCAGAANATGGT

SFC3-3.82

ACCATACTGCTCGAATAAACTGCCTTGCATGGTCTCCTGATAACAGCATGGTCGCTACTGGATCACTTGACACTTGTGTC
ATTATATATGAAGTTGACAAGCCTGCATCGAGCCGTGTAACCATAAAGGGTGCTCATTTGGGCGGGGTATATGGGATAGC
TTTCACCGATGAGCACACAGTGGTAAGCTCTGGCGAGGATGCGTTTGTTCGTGTTTGGAAGTTGACTCCCCAATAGCTTA
TTGTGTANGAATTCAGGAGGTTATTAAAGCATCAATGAAGAAGTTGATGGACTTTCTGGATTTCCTGGTTTTATACTGTT
ACNAGCNTGCCCTGNGTGAGAAAAAAAAAAAATGCANTTTGGAGTTGTTTCTACTTGCTAGTTATAAAACTTGGTGGATTT
CANATGTNATGTTTGTTTCTTAAAAGTCTTTGTTCGATNATTTGAATTTCCCAAANGTCTGAAAAAGT

APPENDIX VII: ALIGNMENTS OF CLONES FROM THE FIRM AND SOFT SUBTRACTIONS.

FFC18

```
gb|AAD10960.1| (AF000521) cell wall invertase precursor [Fragaria x ananassa]
           Length = 577

 Score =  179 bits (450), Expect = 3e-44
 Identities = 90/136 (66%), Positives = 99/136 (72%)
 Frame = -1

Query: 556 KGPLGPFGLMPXVXKGFEXXXSXLXIEFFKSXSXNXEYGFLCAVXXAGLP*TPDNDMXXY 377
           KG LGPFGL+  V K  +   + +    FKS + N +Y  L     +     PDNDM  Y
Sbjct: 443 KGALGPFGLLAFVSKDLKEKTA-IFYRIFKSHNNNNKYVVLMCSDQSRSSLNPDNDMTTY 501

Query: 376 GTFVNVDPLHEXLSXRSLVDHSIVESFGGKGKACIXXRVYPTLAVDGDTHLYAFNYGSES 197
           GTFVNVDPLHE LS RSL+DHSIVESFGGKGK CI  RVYPTLAVDGDTHLYAFNYGSES
Sbjct: 502 GTFVNVDPLHEKLSLRSLIDHSIVESFGGKGKECITARVYPTLAVDGDTHLYAFNYGSES 561

Query: 196 VKIAGSAWSMRXAKIN 149
           VKIAGSAWSM+ AKIN
Sbjct: 562 VKIAGSAWSMKTAKIN 577
```

FFC20

```
sp|P25861|G3PC_ANTMA GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, CYTOSOLIC
           >gi|66012|pir||DESKG glyceraldehyde-3-phosphate
           dehydrogenase (EC 1.2.1.12) - garden snapdragon
           Length = 337

 Score =  152 bits (381), Expect = 4e-36
 Identities = 75/79 (94%), Positives = 79/79 (99%)
 Frame = -2

Query: 238 VPTVDVSVVDLTVRLEKKATYDQIKAAIKEESEGKMKGILGYTEDDVVSTDFIGDNRSSI 59
           VPTVDVSVVDLTVRLEKKATY+QIKAAIKEESEGK+KGILGYTEDDVVSTDF+GD+RSSI
Sbjct: 237 VPTVDVSVVDLTVRLEKKATYEQIKAAIKEESEGKLKGILGYTEDDVVSTDFVGDSRSSI 296

Query: 58  FDAKAGIALNDNFVKLVSW 2
           FDAKAGIALNDNFVKLVSW
Sbjct: 297 FDAKAGIALNDNFVKLVSW 315
```

FFC41

```
gb|AAC32131.1| (AF051230) heat shock protein [Picea mariana]
          Length = 205

 Score = 99.8 bits (245), Expect = 4e-20
 Identities = 49/50 (98%), Positives = 50/50 (100%)
 Frame = +1

Query: 1   TIARSGTKEFMEALAAGADVSMIGQFGVGFYSAYLVAEKVIVTSKHNDDE 150
           TIARSGTKEFMEALAAGADVSMIGQFGVGFYSAYLVAEKVIVT+KHNDDE
Sbjct: 60  TIARSGTKEFMEALAAGADVSMIGQFGVGFYSAYLVAEKVIVTAKHNDDE 109
```

FFC65

```
gb|AAF31705.1|AF221856_1 (AF221856) heat-shock protein 80 [Euphorbia esula]
          Length = 320

 Score =  191 bits (480), Expect = 1e-47
 Identities = 115/187 (61%), Positives = 122/187 (64%), Gaps = 5/187 (2%)
 Frame = +3

Query: 3   HSTKSGDEMTSLKDYVTRMKEGQQDIYYITGESKKAVENSPFLEKLKKKGYXVLFMVDAI 182
           HSTKSGDEMTSLKDYVTRMKEGQ DIYYITGESKKAVENSPFLEKLKKKGY VLFMVDAI
Sbjct: 58  HSTKSGDEMTSLKDYVTRMKEGQSDIYYITGESKKAVENSPFLEKLKKKGYEVLFMVDAI 117

Query: 183 DEYAVGQLKEYEGKKLVSATKEGXXXXXXXXXXXXXXXXXXXXXXXXVVKDVLGDRVEK 362
           DEYAVGQLKE+EGKKLVSATKEG                         V+KDVLGDRVEK
Sbjct: 118 DEYAVGQLKEFEGKKLVSATKEGLKIDESEDEKKKKDELKEQFEGLCKVIKDVLGDRVEK 177

Query: 363 VVXSD-----PCGLILPAVW*LASMAGLRTWXGS*KPXXLXDSTMAGYHGPPXTPWXIQP 527
           VV SD     PC L+       A+M  +       K   L DS+MAGY     T   I P
Sbjct: 178 VVVSDRVVDSPCCLVTGEYGWTANMERIM------KAQALRDSSMAGYMSSKKT-MEINP 230

Query: 528 RXTPIMGGASER 563
              PIM    +R
Sbjct: 231 E-NPIMDELRKR 241
```

FFC89

```
gb|AAC97525.1| (U23066) flavanone 3-hydroxylase [Persea americana]
          Length = 369

 Score = 62.5 bits (149), Expect(3) = 5e-21
 Identities = 30/38 (78%), Positives = 31/38 (80%)
 Frame = -1

Query: 115 HFXXNGRFKNVVHQAVVNSXHSRLSIATFQNPAQEAIV 2
           HF  NGRFKN  HQAVVNS  SR+SIATFQNPA EAIV
Sbjct: 265 HFLSNGRFKNADHQAVVNSNFSRMSIATFQNPAPEAIV 302
```

FFC95

emb|CAA21469.1| (AL031986) cytoplasmatic aconitate hydratase (citrate hydro-lyase)(aconitase)(EC 4.2.1.3) [Arabidopsis thaliana]
Length = 898

Score = 259 bits (654), Expect = 1e-68
Identities = 120/132 (90%), Positives = 126/132 (94%)
Frame = -2

```
Query: 397  VPSGNLYVWDPKSTYIHEPPYFKDMTMSPPGPHGVKNAYCLLNFGDSITTDHISPAGSIH 218
            V SG LY WDPKSTYIHEPPYFK MTMSPPGPHGVK+AYCLLNFGDSITTDHISPAGSIH
Sbjct: 632  VASGTLYEWDPKSTYIHEPPYFKGMTMSPPGPHGVKDAYCLLNFGDSITTDHISPAGSIH 691

Query: 217  KESPAAKYLMERGVDRRDFNSYGSRRGNDEIMARGTFANIRLVNKFLKGEVGPKTIHHPT 38
            K+SPAAKYLMERGVDRRDFNSYGSRRGNDEIMARGTFANIR+VNK LKGEVGPKT+H PT
Sbjct: 692  KDSPAAKYLMERGVDRRDFNSYGSRRGNDEIMARGTFANIRIVNKHLKGEVGPKTVHIPT 751

Query: 37   GDKVSVFDAAMK 2
            G+K+SVFDAAMK
Sbjct: 752  GEKLSVFDAAMK 763
```

FFC104

sp|P37225|MAON_SOLTU NAD-DEPENDENT MALIC ENZYME 59 KD ISOFORM, MITOCHONDRIAL PRECURSOR (NAD-ME) >gi|1076666|pir||A53318 malate dehydrogenase (decarboxylating) (EC 1.1.1.39) 59K chain precursor, mitochondrial - potato >gi|438131|emb|CAA80547.1| (Z23002) precursor of the 59kDa subunit of the mitochondrial NAD+-dependent malic enzyme [Solanum tuberosum]
Length = 601

Score = 131 bits (327), Expect(2) = 7e-38
Identities = 70/103 (67%), Positives = 71/103 (67%)
Frame = +3

```
Query: 87   LXDLGVXGIGXPIGXLDMYVAXAGINPQRVLPVMLDVXTNXXXXXXXXXXXXXRQPRXXG 266
            L DLGV GIG PIG LDMYVA AGINPQRVLPVMLDV TN             RQPR  G
Sbjct: 186 LGDLGVQGIGIPIGKLDMYVAAAGINPQRVLPVMLDVGTNNQKLLEDPLYLGLRQPRLEG 245

Query: 267 XEXLXIVDEFVEAVHARXPXAXVHXEDFQAKXXFETLDRYPEK 395
             E L IVDEFVEAVHAR P A V  EDFQAK  FETLDRY +K
Sbjct: 246 EEYLSIVDEFVEAVHARWPKAVVQFEDFQAKWAFETLDRYRKK 288
```

FFC105

```
gb|AAA86642.1| (U44976) ubiquitin-conjugating enzyme [Arabidopsis thaliana]
          >gi|3746915|gb|AAC64116.1| (AF091106) E2
          ubiquitin-conjugating-like enzyme [Arabidopsis thaliana]
          >gi|6729530|emb|CAB67615.1| (AL132977) E2
          ubiquitin-conjugating-like enzyme Ahus5 [Arabidopsis
          thaliana]
          Length = 160

 Score =  101 bits (250), Expect(2) = 2e-23
 Identities = 49/68 (72%), Positives = 49/68 (72%)
 Frame = +3

Query: 195 GGTIXXXXXXXEDYPSKPPKCKFPQGFFHPNXXPSGTXCLFILNEDKGWEGPPSQVKQIL 374
           GG         EDYPSKPPKCKFPQGFFHPN  PSGT CL ILNED GW  P   VKQIL
Sbjct: 56  GGFFPLTMHFSEDYPSKPPKCKFPQGFFHPNVYPSGTVCLSILNEDYGWR-PAITVKQIL 114

Query: 375 VGIQDLLD 398
           VGIQDLLD
Sbjct: 115 VGIQDLLD 122
```

SFC3-3.S4

```
gb|AAD10327.1| (U63534) cinnamyl alcohol dehydrogenase [Fragaria x ananassa]
          Length = 359

 Score = 50.8 bits (119), Expect = 4e-06
 Identities = 22/22 (100%), Positives = 22/22 (100%)
 Frame = +3

Query: 3   YDGSTTYGGYSDIMVADEHFIV 68
           YDGSTTYGGYSDIMVADEHFIV
Sbjct: 127 YDGSTTYGGYSDIMVADEHFIV 148
```

SFC3-3.1

```
gb|AAF34765.1|AF227620_1 (AF227620) 60S ribosomal protein L10 [Euphorbia esula]
          Length = 220

 Score =  130 bits (324), Expect = 6e-30
 Identities = 59/60 (98%), Positives = 60/60 (99%)
 Frame = +2

Query: 128 YPKSRFCRGVPDPKIRIYDVGMKKKGVDEFPFCVHLVSWEKENVSSEALEAARIACNKYL 307
           YPKSRFCRGVPDPKIRIYDVGMKKKGVDEFPFCVHLVSWEKENVSSEALEAARIACNKY+
Sbjct: 17  YPKSRFCRGVPDPKIRIYDVGMKKKGVDEFPFCVHLVSWEKENVSSEALEAARIACNKYM 76
```

SFC3-3.52

```
gb|AAF34765.1|AF227620_1 (AF227620) 60S ribosomal protein L10 [Euphorbia esula]
          Length = 220

 Score = 76.5 bits (185), Expect(2) = 8e-22
 Identities = 33/36 (91%), Positives = 34/36 (93%)
 Frame = +3

Query: 3   PKSRFCRGVPDPKIRIYDVGMXKKGVDQFPVCVHLV 110
           PKSRFCRGVPDPKIRIYDVGM KKGVD+FP CVHLV
Sbjct: 18  PKSRFCRGVPDPKIRIYDVGMKKKGVDEFPFCVHLV 53
```

SFC3-3.88

```
emb|X13557.1|LE25SRIB Tomato 25S ribosomal RNA gene
          Length = 3381

 Score = 287 bits (145), Expect = 5e-76
 Identities = 148/149 (99%)
 Strand = Plus / Plus


Query: 1    cggctcttcctatcattgtgaagcagaattcaccaagtgttggattgttcacccaccaat 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2877 cggctcttcctatcattgtgaagcagaattcaccaagtgttggattgttcacccaccaat 2936


Query: 61   agggaacgtgagctgggcttagaccgtcgtgagacaggttagttttaccctactgatgac 120
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2937 agggaacgtgagctgggtttagaccgtcgtgagacaggttagttttaccctactgatgac 2996


Query: 121  agtgtcgcaatagtaattcaacctagtac 149
             ||||||||||||||||||||||||||||
Sbjct: 2997 agtgtcgcaatagtaattcaacctagtac 3025
```

SFC3-3.82

```
>dbj|BAB02018.1| (AB020749) WD40-repeat protein [Arabidopsis thaliana]
          Length = 609

 Score = 136 bits (338), Expect = 2e-31
 Identities = 61/77 (79%), Positives = 70/77 (90%)
 Frame = +3

Query: 3   HTARINCLAWSPDNSMVATGSLDTCVIIYEVDKPASSRVTIKGAHLGGVYGIAFTDEHTV 182
           H+ARINCLAWSP+++MVATGSLDTCVI+YEVDKPASSR+TIKGAHLGGVYG+ F D+  V
Sbjct: 533 HSARINCLAWSPNSTMVATGSLDTCVIVYEVDKPASSRMTIKGAHLGGVYGLGFADDSHV 592

Query: 183 VSSGEDAFVRVWKLTPQ 233
           VSSGEDA +RVW  TPQ
Sbjct: 593 VSSGEDACIRVWSFTPQ 609
```

APPENDIX VIII: Sequences of clones identified from the cDNA-AFLP.

Clone Sequence

AFLP-S1

GCCGCCCGGCCCAAAACCCTGGCCCATCATTGGCAACCTCAACCTCATAGGCCATCTACCCCACCGCTCCATCCATACCC
TCTCCCAAAAATATGGTCCCATCATGCAGCTCAAATTCGGGTCTTTCCCGGTCGTCGTAGGATCCTCCGTTGAAATGGCC
AAGGCCTTCCTCAAAACGCATGATGTCACCTTCGCCGGTCGGCCCAAATTTGCCGCCGGCAAACACACCACCTACAACTA
CTCGGACATTACTTGGTCCCCGTACGGCCCGTATTGGCGCCAGGCTCGTAAAATGTGTATCATGGAGCTTTTCAGCAACA
AACGCCTAGAGTCGTACGAGTACATCAGAAGAGAAGANATGGGTGCTTTGCTTANANGCTTGTATGAATCCTCCAACACT
AACGTCTTGCTCAAAGACACCTCTCCACTGTGAGTCTCAATGTTATAAGCCGAATGGTGCTAGGAAAGAANTACACCGAC
NACTCGGAAGATGCGATTGTNAACCCCNGACNAATTCAANAAAATGCTTGATGAACTCTTCTTGCTGAATGGAATTGTTG
AATATTGGGGAATTCAATTCCTTNGGCTN

AFLP-F6A

CATACCAAGCTTCTGATGAACCATTGTATAGTGGATATAGAACCACTATACAATCTTCAACCCAAGAGGAAACCCTGTTA
GACTTTGCACTTTGGGAGCCACCGCATGGCCCCTATAATTCATTCAATTATCCTTGGAGAAATTATGTCAGAGTTGGTGG
TTCTCTGAGGCACTGTGCTTTCATGGTCATGGCAATGCATGGATGCATTCTGTCAGAAATACAGGCACCACCAGAGAAGA
GACAGGTTTTTGCAAATGAGCCCCTGAGGGTTGGTACCGAAGGTGCTAAAGTATTACGTGAGCTANGAAGCAAAGTANAA
AGGATGGAAA

AFLP-F6B

GAAGCAAATGTGGAGGAGTCCCAATGGGACTATTAGGAATATTCTGAATGGTACTGTTTTCAGAGAACCGATTCTTTGCA
AGAACATTCCTCGTCTGATCCCAGGTTGGACGAAGCCAGTATGCATTGGAAGACATGCTTTCGGTGATCAATATCGAGCA
ACTGATACAGTTATCAAAGGACCTGGGAAGCTGAAATTGATGTTTGTTCCAGAGGGAAAGGATGAGAAGATGGAATTCAA
GTAAATGGAGGGAAGTGGCTAAGAAGGAAATTAGTGAAGGGGGGAGCTCATACAAGTGTATTAGTGAATTTGTAGAACTT
CTGGTGCCTGC

AFLP-S7

GCCTCAGAATCAAGTTCTTCTGATAGACGCCAAGGAGCAGATGGATCAGAGACAGAACTAGAATTTGAATTGGAATTCCA
TCTTCTCATCCTGTCCCTCTGGAACAAACATCAGTTTCAGCTTCCCAGGTCCTTTGATAACTGTATCAGTTGCTCGATAT
TGATCACCGAAAGCATGTCTTCCAATGCATATTGGCTTCGTCCAACCTGGGATCAGACGAGGAATGTTCTTGCAAAGAAT
CGGTTCTCTGAAAACAGTACCATTCAGAATATTCCTAATAGTCCCATTGGGACTCCTCCACATTTGCTTC

AFLP-S8

GATGTCACCTTTTACTTCAAGTGAGATGGATGGTGGAAAGGCAGATATTCCTCTGACCACTGTACATGCAAAGCAAGATG
GAGAGAAACCTGTAACATCTCCGTCTACAGCCCGCCCTCCCTTAGAAGAGAATATCATTCTCGGTGTTGCCTTAGAGGGA
TCTAAGCGAACACTTCCAATTGAGGAAGATATGGCTCCATCTCCTATAGAATCAAAAGAATTTACTGCGAGCCGTAATGG
CAGTGGCGGGTCTCCTCTTGGCACAGATTTGAAAGATTGATGACTGCATTCTAGAGCCTGGCC

AFLP-F9A

TGATGCACAACAGGACTTACTGTGCCGAGATTGCACACAACATTTCCACCCGGAAGAGAAAGGAGATTGTTGAACGTGCA
GCTCAGTTGGATATTGTTGTCACCAACAAACTCGCCAGGTTGCGCAGCCAGGAAGACGAGTGAGCTTCAGAGCTATCGTG
GGGATTTCCTTCGTGTGTTTCCTAGTTTCAGTGTTTTGGAAGAGATCTTATTCAGAATCTTATTTTTGTTTTCAATTCAG
CAGTGTTGGATCTAGACTCTAGTCACTAGTTTACTAGTAAGACATGTNAAATGATATG

AFLP-F9B

CAAGCAAGTCAACCTGTCTGGTGAAGCAAGTGAAACGAAAGTTTTGTTGTGCCATAGTCAACCTGTCTGGTGAAGCAAGT
GAAACGAAAGTTTTGTTGTGCCATACGACCTCCTTTTTCAGGTCGCAGGGATGGTCTTCTGTGTTCTCCATTTTGTTTCC
TGATACCATGAGTCTGATGAGGGTTCTGTTTAGAGGTCTAATGAACAGAGAGGAGTTAGTTTGCCCTGCTAGGGATTTAC
TTGTTGTAGTGTGTCCACAGATGGAAACAACGATTGGAATGTAATCAACGTGTACCATTAC

AFLP-S12
GAAACAGCAAAGTCCAGAAAACGTGTTTCTAACAAGACCTGAATTCCATCATCTGTGCTTTTCTTCAGATCTGGATGGTT
GAACTCATTTTGAGGCAAGACAATGAGTTGGCCCTTGCCATGACCTTTTTCAAGAGCCCATCCGCTGTTGGCTACATGAT
GCTCAAGGAATTTGTCCAAGGATATACCTTCAAGGTTGATTGCCTCAGCAAGCACANGTCTGGGAACCTTTTGGT

AFLP-S13
GTTAGTCTGATGAATACTATCGTGTGAGATGGAAGTCTGGAACTGTCACTAGCAAAGTTCCTTGTTGTGCAATCACACTA
ATTTTTTTTCCTGTTGCCTCGTTTCCAGAATAAATTACAAATGTGTTTAGCTTTTAGAAGAAATATAATTTCAACATTGT
TCAAACCTCTCCCTCCTCCTCTTGTTCTGGTTTGGTTGCATGTGGTAGACACAAAGCCAATGACAGTTGTA

AFLP-S14A
GACGAGACTTTCGTTTCTGCGTGATGCTAGTGATCNAGTTGAATTGGAGGATAGAGAGACTGCATCTCAACTCTTAAGTT
CATTAGAAAGTACCGCATCAGTTGCTCTAAATGGAAAGTGGGATGAAAAGATGGAGCCTGCATTCCTCAATAACATTGGT
CGCTACAGGCGTTACAAGTTTGACAGCATCCGTGACTTGCTGCGTGTAAT

AFLP-S14B
TCANCANCTGATACNACTGAAGCCAAATAAGGANACTCTGGNNCTACACATCTTCCANGCTGAGTTATGTTGTTTTTCCA
GCCACACNAACATGTTGGGCAGGGAACTACTGTGTCATTGTACAAGGAANACANTNANACTCANCAANGATGATCTTTCT
GATCCANGAACTGTGAATATGTGCATGTCACATTCNATNTCNCTNCCACCAA

AFLP-S15
GAAACAGCAAANTGCAGAAAACGTGTTTCTAACAAGCCCTGAATTCTANCTGGGGGTGCAGCTGCTGCTGCAGCCGCATA
CGGTGCTCACCAATTGACAGGCCATCACGGTCAAGGCTCTCACCAAGTTTCGCACGGCGGCTACAATCCATCTCACGGCA
TGCCAGGGTACGGGGGTCACGGCATGCCATGGTACGGGGGTCACGGC

AFLP-F16
NCCCCAGAATCAANTTCTTCTGATAGACGCCAAGGAGCAGATGGATCANANACAGAACTANAATTTGAATTGGAATTCCA
AACNTTTATCATTTCGGACTANAGTGANAGATGAGACCCTCNAGTTCAAGTATAATGGTGGCAAGANCATTACATANTTC
AGATTTGGGTCTCTCAACANAATATCCGA

AFLP-F18
TCTGAAACAACAAAAATGAAAACTGAAAATTGATCACACTCTCGCTTCAAAAGAGTTCCACGCTTACCCTACATTTTGGC
GCTTCAAAACCAAATTAGAATTCAACATACAAAAGAGAGCAGCAGAAAGAAAAGAAAGGCCCTACTCGTCTGAAGGCAAC
TTC

AFLP-F19
GGAGGGCAGGCTTATCCTATTGTTGAAGGAGAGGGGAATGCTGGGAAGAANACAAAGATAACTGGGAATTCCAATTCAAA
TTCTAGTTCTGTCTCTGATCCATCTGCTCCTTGGCGTCTATCAGAANAACTTGATTCTGGGGCT

AFLP-S20A
TAGGGGTTTTTCTAGTAAAAGAAAAGGAGGGAGCACTGGGGATTTGAGAAGAGGAGGAGGGAGGGGAAGGTGAAGATCTG
GCTTTCTCGTTTTGAAACATTTTTCATTCGATCGATCGAGGATATATGGCTGCTGGGGACGAT

AFLP-S20B
ACAACGGTTGCTGCGAGAAAAAATTACCACTACCTTCAGTTTATCCATTTTCTCCACAAATTCGGGCAAGGCATATCTGT
ATGTATCGAAATTCAAGACAAGAACCTCTGCTTCAGGTAGTTGAATCTGGGGCCATTTCATT

AFLP-S21A
GCTATCGTTTACTCCTGGATTTCTTGTGAGTCTATGGGGAGCGTTGGAAACCTATCTCTTTCCTAGGATTGTTTGCAGTG
GTCATAAACCCTATGATAACATTAGTAAAACTTCTGGTAGTGGAAAGGAT

AFLP-S21C
AGCTTTGGAAAGTCCAATTCATCCATAATCTCNCAGCTAGCTTCATCAGGAAAAGTAAAGAANATGTTTGCTCACTGCTT
AGATGGAAAAAATGGAGGCGGTATCCTTGCTATTGTGCGTGTTGTGCAGTCAA

AFLP-S22B
ACCCAATCCACAAGGCCTACAAAATTCTCNTTTGGTCGAAACTTCATGTTGTTCTTTCCANCAACTATTTCCAGTGCGAC
AACTCCAAAACTGAAGACATCTGCTTTG

AFLP-S22C
ACAGAAAATACATCTATGAACAGGAAATNNTTGGTTTNCTAACTAATCTGAGAAGCCGAAACATAAAGCCTACCCATCTG
GTTAGANATATCCAACTCCATAA

AFLP-F24
GTGAGACAAACGCGCGCTTCTGATTTGGGTTTTTGGGGTTCTTGTGGCCTCTGGATGACCCAAGGATAGTCT

AFLP-S25
AGTCTTGAGTTTGATAACGAAAATGGATTNNACNATCTAGCTAAGCAGTAAACGGCGCCGGAT

AFLP-S26
AGATATGCNGTACANATTCATTGATCTNCNCTTNNAATGATTCAAGTCTTGCACCATAATATCTTTGCACTGTCCACCCT
ATGTGAAACTATTCTTCCGCCACTGANAGGGTAAGCATCCATGGCTGGATTTGAAAGCCCAAAACATATGAAATGAGCTG
GAATTGCTGCGGTACAAACCGA

AFLP-F26B
AGACTGAACTGGCTGTTTGGGCACCCATAGGACTTTATGTATATAGTTATGCTCTCCTGCATACAAACATTGTGATATAT
AATTATCTATATGGTATATTCATAGCTCAAGTTGTAACTGGTTTTGAAATGCGGTCAGGTGANTAATGATTGATGTGCAC
TGCCGCTTTTGGAATTGTTC

AFLP-F26D
GACTGAACTGGCTGTTTGGGCACCCATAGGACTTTGTGTATATAGTTATGCTCTCCTGCATACAAACATTGTGATATATA
ATTATCTATATGGTATATTCATAGCTCAAGTTGTAACTGGTTTTGAAATGCGGTCAGGTGAGTAATGATTGATGTGCACT
GCCGCTTTTGGAATTGTTC

APPENDIX IX: ALIGNMENTS OF CLONES FROM THE FIRM AND SOFT cDNA-AFLP.

AFLP-S1

```
>pir||T03275 probable cytochrome P450, hypersensitivity-related - common tobacco
 emb|CAA64635.1| (X95342) cytochrome P450 [Nicotiana tabacum]
          Length = 509

 Score =  213 bits (536), Expect = 1e-54
 Identities = 107/187 (57%), Positives = 132/187 (70%), Gaps = 1/187 (0%)
 Frame = +2

Query: 2   PPGPKPWPIIGNLNLIGHLPHRSIHTLSQKYGPIMQLKXXXXXXXXXXXXEMAKAFLKTH 181
           PPGPKPWPIIGNLNLIG+LP+RSIH LS KYGP+MQL+             EMAK FLK+
Sbjct: 34  PPGPKPWPIIGNLNLIGNLPYRSIHELSLKYGPVMQLQFGSFPVVVGSSVEMAKIFLKSM 93

Query: 182 DVTFAGRPKFAAGKHTTYNYSDITWSPYGPYWRQARKMCIMELFSNKRLESYEYIRREXM 361
           D+ F GRPK AAGK+TTYNYSDITWSPYGPYWRQAR+MC+ ELFS K L+SYEYIR E +
Sbjct: 94  DINFVGRPKTAAGKYTTYNYSDITWSPYGPYWRQARRMCLTELFSTKCLDSYEYIRAEEL 153

Query: 362 GALLXXLYESSNTNVLLKD-TSPL*VSML*AEWC*ERXTPTTRKMRLXTPDXFXKMLDEL 538
            +LL  L + S   ++LKD  + L ++++      +R    +       P+ F KMLDEL
Sbjct: 154 HSLLHNLNKISGKPIVLKDYLTTLSLNVISRMVLGKRYLDESEN-SFVNPEEFKKMLDEL 212

Query: 539 FLLNGIV 559
           FLLNG++
Sbjct: 213 FLLNGVL 219
```

AFLP-F6A

```
>gb|AAD49983.1|AC008075_16 (AC008075) F24J5.16 [Arabidopsis thaliana]
          Length = 533

 Score =  178 bits (447), Expect = 2e-44
 Identities = 78/109 (71%), Positives = 95/109 (86%)
 Frame = +3

Query: 3   YQASDEPLYSGYRTTIQSSTQEETLLDFALWEPPHGPYNSFNYPWRNYVRVGGSLRHCAF 182
           YQASD+PLYSGYR+ +QS++QE++LLDFA+WEPPHGPY +FN+PW+NYV++ G++RHCAF
Sbjct: 256 YQASDDPLYSGYRSAVQSTSQEDSLLDFAIWEPPHGPYKTFNHPWKNYVKLSGAVRHCAF 315

Query: 183 MVMAMHGCILSEIQAPPEKRQVFANEPLRVGTEGAKVLRELXSKVXRME 329
            VMAMHGCILSEIQA PEKR VF+NE  RVG EGAKVLR    KV +ME
Sbjct: 316 TVMAMHGCILSEIQASPEKRHVFSNELRRVGNEGAKVLRLFGEKVEKME 364
```

AFLP-F6B

```
>gb|AAD51361.1|AF176669_1 (AF176669) NADP-isocitrate dehydrogenase [Citrus limon]
          Length = 414

 Score =  163 bits (408), Expect = 6e-40
 Identities = 71/80 (88%), Positives = 78/80 (96%)
 Frame = +2

Query: 2   KQMWRSPNGTIRNILNGTVFREPILCKNIPRLIPGWTKPVCIGRHAFGDQYRATDTVIKG 181
           KQMW+SPNGTIRNILNGTVFREPI+CKN+PRLIPGWTKP+CIGRHAFGDQYRATDTVI+G
Sbjct: 91  KQMWKSPNGTIRNILNGTVFREPIICKNVPRLIPGWTKPICIGRHAFGDQYRATDTVIQG 150

Query: 182 PGKLKLMFVPEGKDEKMEFK 241
           PGKLKL+FVPEGKDEK E +
Sbjct: 151 PGKLKLVFVPEGKDEKTELE 170
```

AFLP-S7

```
>gb|AAD51361.1|AF176669_1 (AF176669) NADP-isocitrate dehydrogenase [Citrus limon]
          Length = 414

 Score =  163 bits (408), Expect = 6e-40
 Identities = 71/80 (88%), Positives = 78/80 (96%)
 Frame = +2

Query: 2   KQMWRSPNGTIRNILNGTVFREPILCKNIPRLIPGWTKPVCIGRHAFGDQYRATDTVIKG 181
           KQMW+SPNGTIRNILNGTVFREPI+CKN+PRLIPGWTKP+CIGRHAFGDQYRATDTVI+G
Sbjct: 91  KQMWKSPNGTIRNILNGTVFREPIICKNVPRLIPGWTKPICIGRHAFGDQYRATDTVIQG 150

Query: 182 PGKLKLMFVPEGKDEKMEFK 241
           PGKLKL+FVPEGKDEK E +
Sbjct: 151 PGKLKLVFVPEGKDEKTELE 170
```

AFLP-F9A

```
>pir||T14810 ribosomal protein L32, cytosolic - Arabidopsis thaliana
 emb|CAB53651.1| (AL110123) ribosomal protein L32-like protein [Arabidopsis
          thaliana]
 emb|CAB78812.1| (AL161547) ribosomal protein L32-like protein [Arabidopsis
          thaliana]
          Length = 133

 Score = 87.4 bits (213), Expect = 4e-17
 Identities = 40/46 (86%), Positives = 45/46 (96%)
 Frame = +3

Query: 3   MHNRTYCAEIAHNISTRKRKEIVERAAQLDIVVTNKLARLRSQEDE 140
           MHNRTYCAEIAHN+ST+KRK IVERA+QLD+VVTN+LARLRSQEDE
Sbjct: 88  MHNRTYCAEIAHNVSTKKRKAIVERASQLDVVVTNRLARLRSQEDE 133
```

AFLP-S12

```
>pir||T05976 hypothetical protein F17M5.10 - Arabidopsis thaliana
 emb|CAB38783.1| (AL035678) putative protein [Arabidopsis thaliana]
 emb|CAB80042.1| (AL161583) putative protein [Arabidopsis thaliana]
          Length = 226

 Score = 88.2 bits (215), Expect = 1e-17
 Identities = 41/64 (64%), Positives = 51/64 (79%)
 Frame = -3

Query: 233 QKVPRXVLAEAINLEGISLDKFLEHHVANSGWALEKGHGKGQLIVLPQNEFNHPDLKKST 54
           QKVPR VLAEA+N++G SLDKF+E  V NSGW +EK    +G  IVLPQNEFNHP+LKK+T
Sbjct: 153 QKVPRSVLAEAVNMDGASLDKFIEQQVTNSGWIVEK---EGGSIVLPQNEFNHPELKKNT 209

Query: 53  DDGI 42
            + +
Sbjct: 210 GENV 213
```

AFLP-S14A

```
>gb|AAD32909.1|AC007584_7 (AC007584) putative protein kinase/endoribonuclease
[Arabidopsis
          thaliana]
          Length = 393

 Score =  109 bits (271), Expect = 3e-24
 Identities = 51/68 (75%), Positives = 60/68 (88%)
 Frame = +2

Query: 5   RLSFLRDASDXVELEDRETASQLLSSLESTASVALNGKWDEKMEPAFLNNIGRYRRYKFD 184
           RLSFLRDASD VELE+RE  S++L ++ESTA VA+ GKWDEK+EP F+ NIGRYRRYK+D
Sbjct: 262 RLSFLRDASDRVELENREADSEILKAMESTAPVAIGGKWDEKLEPVFITNIGRYRRYKYD 321

Query: 185 SIRDLLRV 208
           SIRDLLRV
Sbjct: 322 SIRDLLRV 329
```

AFLP-S14B

```
>emb|CAA07251.1| (AJ006787) putative phytochelatin synthetase [Arabidopsis thaliana]
          Length = 362

 Score = 79.6 bits (193), Expect = 4e-15
 Identities = 34/62 (54%), Positives = 43/62 (68%)
 Frame = -1

Query: 194 MVTCTYSQFXDQKDHXX*VXXXSLYNDTVVPCPTCXCGWKNNITQXGRCVXPEXPYLASV 15
           N+TCTYSQF  Q+     V   S YN+T+V CPTC CG +NN T+ G C+ P+ P+LASV
Sbjct: 123 NITCTYSQFLAQRTPTCCVSLSSFYNETIVGCPTCACGCQNNRTESGACLDPDTPHLASV 182

Query: 14  VS 9
           VS
Sbjct: 183 VS 184
```